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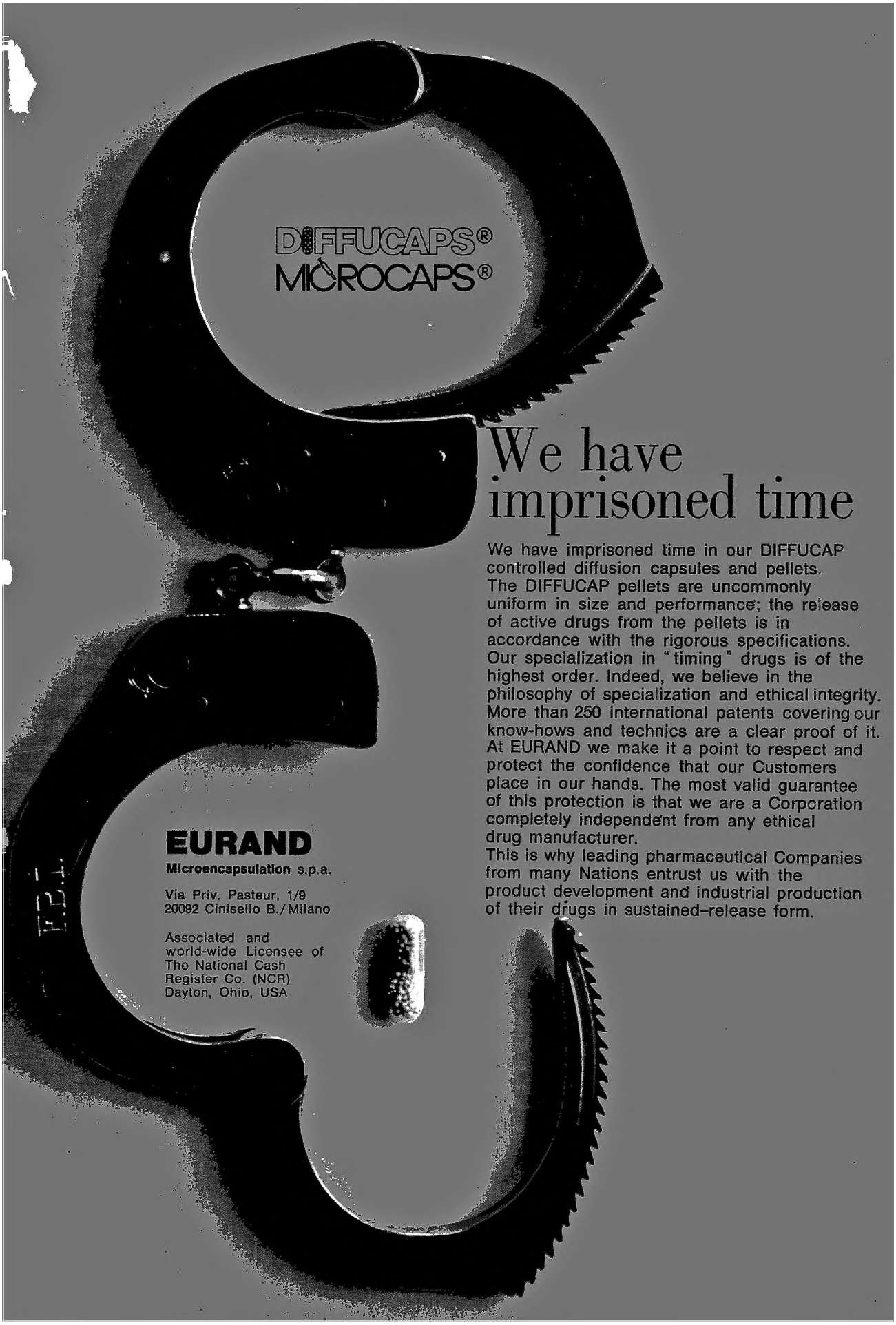

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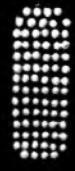

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The relation between circulating and tissue concentrations of salicylate in the mouse *in vivo*

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The blood and water contents of mouse liver, brain, kidney, heart, spleen and skeletal muscle were measured and used to correct observed values for the salicylate concentrations in these tissues after the intraperitoneal injection of the drug. The binding of salicylate *in vitro* to mouse whole blood, liver, kidney and brain was studied. It was concluded that blood, liver and kidney but not the other tissues, bind the drug *in vivo*.

Salicylate inhibits the activity of several enzyme systems *in vitro* including oxidative phosphorylation reactions, dehydrogenases, aminotransferases (Smith, 1968), nucleic acid polymerases (Janakidevi & Smith, 1969) and aminoacyl-t-RNA synthetases (Burleigh & Smith, 1970). The mechanisms of many of these inhibitory actions of the drug involve competition between the salicylate and either a substrate or coenzyme (Gould, Dawkins & others, 1966; Dawkins, Gould & others, 1967). An important factor is therefore the concentration of salicylate in the reaction mixtures and *in vitro* this is easily controlled and remains constant during the experiment. To explore the possible relevance of *in vitro* inhibitions to *in vivo* effects it is necessary to show that they both occur at equivalent concentrations. The tissue concentrations of salicylate occurring after the administration of the drug must be known to make such comparisons of any value. Sturman, Dawkins & others (1968) attempted to provide suitable data for the mouse by measuring the total salicylate concentrations in several tissues at varying times after the intraperitoneal injection of a range of doses of salicylate. However, their results showed no apparent correlation between the concentrations in the individual tissues. The present work describes certain correction factors concerned with the volumes of blood sequestered in the tissue samples removed for analysis, the water contents of the tissues and the degree of binding of salicylate to blood and tissue proteins.

EXPERIMENTAL

Blood content of tissues

Male mice, 25–30 g, of the albino strain maintained at King's College Hospital on M.R.C. modified cube diet no. 41B were used in this and in the subsequent experiments. Eight mice each received an intravenous (tail-vein) injection (0·2 ml) containing 10 μCi of ^{131}I-labelled human serum albumin (specific activity, 30 μCi/mg albumin, obtained from the Radiochemical Centre, Amersham, Bucks) in 0·9% (w/v) NaCl and were killed after 8 min by cervical fracture. Blood samples were collected, after decapitation, into lithium heparin tubes, and the liver, brain, kidneys, heart, spleen and both quadricep femoralis muscles from each animal were rapidly removed, weighed on a torsion balance, digested in 1 ml of 30% (w/v) NaOH in a

boiling water bath, cooled and made up to 20 ml with distilled water. Aliquots (0·1 ml) were dried on Whatman GF/A (2·1 cm) glass fibre discs (Gallenkamp & Co. Ltd.) and the radioactivity counted in a Beckman LS 200B liquid scintillation system using as phosphor 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-2-(5-phenyloxazolyl)benzene in toluene. The results were calculated as percentages of blood (ml) per 100 g wet weight of tissue by dividing the radioactivity (counts/min) per 100 g wet weight of tissue by the radioactivity per ml of blood.

Water content of tissues

The liver, brain, kidneys, heart, spleen and muscles were removed from four mice, weighed in tared containers and heated at 100° to constant weight. The loss in weight was taken as the water content and calculated as the percentage of water (ml) for 100 g wet weight of tissue.

Binding of salicylate to whole blood

Three groups, each of twelve mice, were killed by cervical fracture and the blood, obtained after decapitation, from each group was pooled during collection in lithium heparin tubes. Aliquots (1 ml) from each pooled sample were placed inside dialysis sacs of Visking tubing (8/32 inch inflated diameter, obtained from the Scientific Centre, London) and dialysed against 3 ml of 0·9% (w/v) NaCl solution, containing sufficient sodium salicylate (British Pharmacopoeial grade) to give initial salicylate concentrations ranging from 0 to 5mM, in vessels shaken 100 cycles/min for 24 h either in a water bath at 8° or at room temperature (22°). Salicylate was determined in samples taken from the fluid outside the sacs with an Aminco Bowman spectrophotofluorometer, using an activating wavelength of 294 nm and a detecting wavelength of 413 nm. The salicylate concentration outside the sacs at the end of the dialysis is the unbound concentration. Some salicylate disappears during dialysis due, at least in part, to adsorption onto the Visking tubing. The amount of salicylate added at the beginning of the experiments is therefore not equal to the amount remaining at the end. The amount of salicylate inside the sac containing the homogenate was calculated by subtracting the amount outside the sac at the end of the dialysis from the total amount, i.e. inside and outside the sac, found in similar experiments in which saline had been substituted for the tissue homogenate.

The tissues were removed from 20 mice and individually homogenized in an all-glass Potter homogenizer with an equal volume of 0·9% (w/v) NaCl solution. In some experiments the livers were homogenized without saline. Samples (1 ml) of the homogenates were placed inside Visking dialysis sacs and treated as described above for the whole blood experiments except that additional experiments were made with the liver homogenate in which 0·1 μCi of [^{14}C]carboxyl salicylic acid (specific activity 31·4 mCi/mmol obtained from the Radiochemical Centre) was added to the unlabelled salicylate. Salicylate was determined in samples taken from the fluid outside the sacs and the radioactivity measured in these samples after dialysis.

RESULTS

The percentage contents of blood and water in mouse liver, brain, kidney, heart, spleen and skeletal muscle are given in Table 1. The concentrations of total and

Table 1. *Contents of blood and water in mouse tissues.* Each value represents the mean ± standard deviation and is expressed as ml of either blood or water in 100 g wet weight of tissue.

Tissue	% blood in tissue (8 animals)	% water in tissue (4 animals)
Liver	17·2 ± 2·8	71·6 ± 1·0
Brain	2·6 ± 0·8	79·5 ± 0·8
Kidney	14·8 ± 3·4	76·3 ± 1·0
Heart	22·0 ± 4·0	79·4 ± 1·4
Spleen	13·8 ± 2·0	79·5 ± 1·2
Muscle	2·7 ± 0·8	73·7 ± 1·8

Table 2. *Binding of salicylate to mouse blood.* Each value is given as the mean ± standard deviation of duplicate determinations made on two of the pooled samples of mouse blood.

Salicylate concentration (mM)		
Initially present outside sac before dialysis	Found outside sac at end of dialysis (Unbound concentration)	Calculated as present inside sac at end of dialysis (Total concentration)
0·05	0·03 ± 0·001	0·07 ± 0·002
0·10	0·05 ± 0·001	0·14 ± 0·007
0·50	0·28 ± 0·003	0·60 ± 0·006
1·00	0·60 ± 0·027	1·12 ± 0·065
1·50	0·99 ± 0·008	1·52 ± 0·028
2·00	1·29 ± 0·014	2·06 ± 0·056
5·00	3·11 ± 0·064	5·28 ± 0·153

Table 3. *Unbound and total salicylate in mouse tissues* in vitro. Experimental conditions as in text, each value is the mean of four determinations and is expressed as in Table 2.

Salicylate concentration (mM)					
Brain		Liver		Kidney	
Unbound	Total	Unbound	Total	Unbound	Total
0·04	0·04	0·03	0·07	0·03	0·06
0·09	0·08	0·06	0·12	0·06	0·13
1·29	1·29	0·64	1·09	0·66	1·02
		1·02	1·43	1·06	1·35
		1·36	1·91	1·46	1·62

unbound salicylate in mouse blood exposed at 8° to salicylate concentrations, ranging from 0·1 to 5·0mM, are given in Table 2. Three different samples of mouse blood were used in the experiments, each salicylate concentration being tested against two of these. The percentage of unbound salicylate increased with the total salicylate concentrations in whole blood, being approximately 40% at 0·1 mM and 60% at about 5 mM. The results of similar experiments with homogenates of brain, liver and kidney are given in Table 3. Similar results with the whole blood and the tissues were observed when the dialyses were performed at either 8° or 22°. There

was no difference between the free and total concentrations for brain and this tissue did not therefore bind the drug. However, both liver and kidney showed an apparent binding in that the calculated total salicylate concentrations inside the sacs exceeded the free concentrations measured in the fluid outside the sacs at the end of the dialysis. Similar results were observed with liver homogenates prepared either with or without saline. A further set of experiments was therefore made with mouse liver homogenates using unlabelled salicylate plus a tracer amount of radioactive salicylate. The salicylate concentration and the radioactivity in the fluid outside the sacs were measured at the end of the dialysis. The concentration of salicylate decreased in a similar manner to that shown in Table 3 and this was paralleled by the decrease in radioactivity so that the specific activity remained constant during the dialysis. This result excluded the possibility that salicylate conjugates, which were freely permeable but lacked the characteristic fluorescence of salicylate itself, had been formed. Either salicylate was bound to the liver material or salicylate conjugates were synthesized and subsequently bound to liver proteins.

DISCUSSION

In earlier work (Sturman & others, 1968) the total salicylate concentrations in several mouse tissues, including whole blood, were measured at varying time intervals after the injection of a range of doses of the drug. These data need to be corrected for several reasons. Firstly, the amounts of blood contained in the tissue samples taken for analysis were not taken into account. Secondly, it was assumed that the salicylate was distributed throughout the whole of the tissue sample. Thirdly, the ratios between the protein-bound and unbound drug in the blood were unsatisfactory because insufficient time had been allowed for equilibration in the dialysis method. Finally, no attempt was made to investigate if one or more of the selected tissues could bind salicylate. The present work supplies appropriate correction factors for the blood and water contents of the tissues, allows the percentages of unbound salicylate in the blood to be calculated, and suggests that two of the tissues, liver and kidney, can bind the drug.

The original data of Sturman & others (1968) for the time interval, 30 min after injection, when maximum blood and tissue salicylate concentrations were attained have been recalculated to include the corrected tissue concentrations and free salicylate levels in the blood (Table 4). The recalculated figures represent the concentrations

Table 4. *Distribution of salicylate in mouse tissues.* The values are the data of Sturman & others (1968) for the samples taken 30 min after injection, corrected for blood and water contents of the tissues (see Table 1) and with the concentrations of unbound salicylate in the blood calculated from the results in Table 2.

Dose injected (mg/kg)	Blood		Salicylate concentration (mM)					
	Total	Unbound	Brain	Heart	Spleen	Muscle	Liver	Kidney
50	0·32	0·16	0·06	0·05	0·05	0·07	0·15	0·29
100	0·64	0·35	0·18	0·16	0·16	0·13	0·48	0·46
200	1·09	0·62	0·39	0·40	0·44	0·35	1·20	0·72
400	2·42	1·41	0·92	1·08	1·10	0·98	2·15	1·91
800	3·78	1·94	1·70	1·42	1·58	2·22	2·34	3·10

of salicylate in the combined intracellular and interstitial fluid spaces of the tissues and not the concentrations of the drug in individual cells. The tissues may be divided into two main groups with respect to the relation of their tissue salicylate concentrations and the concentration of unbound salicylate in the blood. The first group comprises brain, skeletal muscle, heart muscle and spleen. At each dose level in each tissue the tissue salicylate concentration is less than the unbound salicylate in the blood suggesting that none of these tissues bind the drug *in vivo*. The results of the *in vitro* binding experiments with mouse brain (Table 3) are consistent with this view. However, as the size of the injected dose is increased so the tissue salicylate concentrations become closer in value to the corresponding free salicylate concentrations in the blood. One explanation of this effect is that with increasing concentration of free salicylate in the blood there is a more rapid equilibration between the blood and the tissues. Thus, during the period at which the blood salicylate concentration is rising after absorption, the drug accumulates in the tissues at a rate proportional to the free salicylate concentration in the blood and hence indirectly to the size of the administered dose. This effect did not occur in the liver and the kidney. In both tissues the salicylate level exceeded the free salicylate concentration in the blood suggesting that either the tissues bound approximately 40% of the drug to intracellular macromolecules or that salicylate conjugates accumulated within the tissues. The results of the *in vitro* experiments (Table 3) show that binding occurs, the figures for bound salicylate again being approximately 50% of the total. Liver and kidney are the two tissues in which salicylate persists for over 18 h after the intraperitoneal injection of the drug in the mouse (Sturman & others, 1968) and this effect is explicable if protein binding of the drug is restricted to them.

Acknowledgement

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Adrenergic neuron blocking properties of (±)-propranolol and (+)-propranolol

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The action of (±)-propranolol and (+)-propranolol on the electrical stimulation of adrenergic nerves to smooth muscle has been studied in the isolated ear artery from rabbits and the isolated vas deferens preparation from rats. Both drugs exhibited an adrenergic neuron blocking action at a pre-junctional site at concentrations ranging from 4·6 to 14 μg/ml. At lower concentrations the effects were variable and more often potentiation of the responses was observed. The responses to added noradrenaline were uniformly potentiated. The effect was related to local anaesthetic activity and not considered to be a specific adrenergic neuron blocking effect as occurs with guanethidine or bretylium.

The observed hypotensive effects of propranolol in man (Prichard & Gillam, 1969; Zacharias & Cowan, 1970) cannot, at present, be accounted for by a convincing pharmacological explanation. Possible mechanisms involving a reduction in cardiac output (Frohlich, Tarazi & others, 1968) or a re-setting of baro-receptors (Prichard & Gillam, 1969) have yet to be substantiated experimentally. For this reason, the investigations of Day, Owen & Warren (1968) comparing the pre-synaptic adrenergic neuron blocking actions of guanethidine and propranolol were of special interest. They demonstrated that guanethidine was three times more potent than propranolol on the rat vas deferens preparation and that the two drugs were equipotent in the rabbit isolated ear artery preparation. (The doses recommended for the treatment of hypertension are 30–60 mg daily for guanethidine and 240–300 mg daily for propranolol.)

In addition to its specific adrenergic β-receptor blocking properties (Black, Crowther & others, 1964), propranolol also possesses a powerful local anaesthetic action equivalent to that of lignocaine (Morales-Aguilera & Vaughan Williams, 1965). A comparison of the isomers of propranolol showed that the (−)-isomer was at least 100 times more active than the (+)-isomer in antagonizing β-receptor stimulation whereas the isomers were indistinguishable in terms of local anaesthetic effects (Barrett & Cullum, 1968). It was important, therefore, to confirm the findings concerning adrenergic neuron blockade and to define the properties of (+)-propranolol in this respect.

EXPERIMENTAL

The technique for the rabbit isolated ear artery preparation was as described by De la Lande & Rand (1965). The rat isolated vas deferens was studied by the same technique as Day & others (1968) with the exception that the duration of the

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stimulus was 1 ms, the frequency 2–10 pulses/s and stimulation maintained for 5 s, repeated at 3 min intervals.

The drugs used were propranolol hydrochloride, (+)-propranolol, practolol (ICI) and guanethidine sulphate (Ciba). All concentrations are expressed in terms of the base.

RESULTS

Rabbit isolated ear artery preparation. At a concentration of 10 μg/ml propranolol produced a highly significant decrease (91% ± 4%; mean ± s.e.; n = 6) in the pressor responses to electrical stimulation. The onset of inhibitory action was always within 10 min and recovery was rapid following washing out of the drug. Similar results were obtained with (+)-propranolol at 10 μg/ml, the mean inhibition being 63 ± 16% (n = 5), a value which was not significantly different, statistically, from that obtained with the racemic compound. In the presence of both drugs the response to extraluminal noradrenaline (0·1–0·4 μg/ml) was always potentiated (Fig. 1).

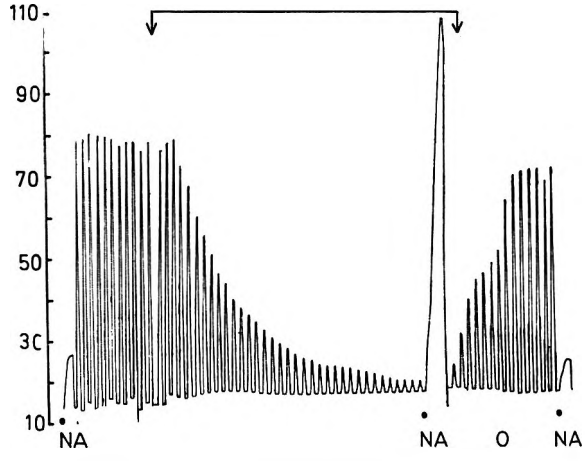

FIG. 1. Responses of rabbit isolated ear artery to electrical stimulation (10 pulses/s for 15s every 2 min) and to noradrenaline (0·1 μg/ml) in the absence and presence (between the arrows) of (+)-propranolol (10 μg/ml).

At lower concentrations (0·1–5 μg/ml) propranolol did not exert a consistent effect on the responses to electrical stimulation. From a total of 22 preparations, inhibition was only observed five times whereas potentiation of the response was present on 13 occasions and no effect on four. The effects were not clearly dose related since both 0·1 and 5 μg/ml produced potentiation and at 3 μg/ml four preparations were inhibited and two potentiated. At these concentrations the response to noradrenaline was potentiated in most preparations. Comparable experiments with guanethidine (0·5–1·0 μg/ml) demonstrated a 75–100% inhibition of the responses to electrical stimulation (Fig. 2). From these experiments it was calculated that guanethidine was 6–20 times more potent than propranolol or (+)-propranolol in producing adrenergic neuron blockade.

Rat isolated vas deferens preparation. In preliminary experiments it was found that in this preparation also the effects of propranolol were substantially less than

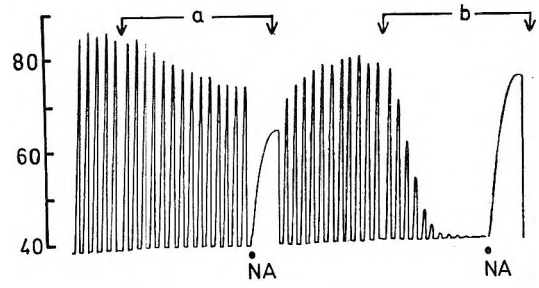

FIG. 2. Responses of rabbit isolated ear artery to electrical stimulation (10 pulses/s for 15 s every 2 min) and noradrenaline (0·2 μg/ml) in the presence and absence of (a) (±)-propranolol (3 μg/ml) and (b) guanethidine (1 μg/ml).

those of guanethidine (Fig. 3). In the presence of propranolol the response to noradrenaline (0·5 μg/ml) was clearly potentiated. Raising the concentration of propranolol produced a dose-dependent inhibition of the responses to electrical stimulation. The onset of blockade was within 5 min but recovery was variable with the exception of the highest concentration when it was uniformly rapid (Fig. 4). The effects of (+)-propranolol were quantitatively and qualitatively similar. From experiments in 15 preparations the mean concentration of propranolol required to produce a 50% inhibition of the contractions was 14 μg/ml. At the lower concentration of both propranolol and its (+)-isomer a modest potentiation of the response to electrical stimulation was observed on two occasions for each drug. From

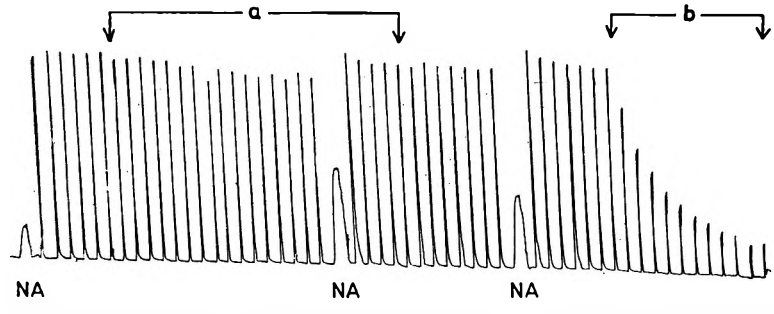

FIG. 3. Responses of rat isolated vas deferens to electrical stimulation (5 pulses/s for 5 s every 3 min) and noradrenaline (0·5 μg/ml) in the presence and absence of (a) (±)-propranolol (4 μg/ml) and (b) guanethidine (1 μg/ml).

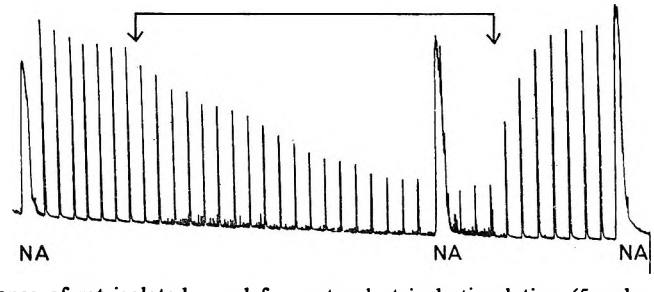

FIG. 4. Response of rat isolated vas deferens to electrical stimulation (5 pulses/s for 5 s every 3 min) and noradrenaline (0·5 μg/ml) in the presence and absence of (±)-propranolol (20 μg/ml).

these experiments it was calculated that guanethidine was 20 times more potent than propranolol. Practolol (50 μg/ml) did not significantly alter the responses of the vas deferens to electrical stimulation.

DISCUSSION

Contractions of rat isolated vas deferens or rabbit isolated ear artery preparations are inhibited by (±)-propranolol and (+)-propranolol when stimulation is electrical via nervous tissue but not when due to added noradrenaline. Such a pre-junctional blocking action is also showed by xylocholine, bretylium and guanethidine. As occurs with these three agents, the responses to exogenous noradrenaline are potentiated by propranolol. It has been suggested by Day & others (1968) that propranolol possesses a potent blocking action on adrenergic sympathetic neurons in isolated smooth muscle comparable to that of guanethidine with the exception that unlike the blockade produced by bretylium or guanethidine, reversal by amphetamine (Day, 1962) is not apparent in the case of propranolol. Three important differences emerged between the results of the present experiments and those of Day & others (1968). First, the potency of propranolol in our hands was 6–20 times less than that of guanethidine whereas results with the latter substance were similar to those of Day & others (1968) and many other workers. Second, the duration of blockade following washing out of the drug was short and not prolonged. Third, at concentrations below those necessary to produce blockade, potentiation of responses to electrical stimulation was frequently seen with both (±)- and (+)-propranolol. The concentrations of propranolol required to produce a 50% decrease in electrically stimulated contractions ranged from 4·6 to 14·0 μg/ml whereas the concentration found necessary to produce a 50% block of conduction in frog isolated sciatic nerves was about 20 μg/ml (Barrett & Cullum, 1968). There was therefore no great difference in the concentrations required to produce evidence of adrenergic neuron blockade or conduction block in motor nerves.

The effective concentrations of propranolol in isolated smooth muscle preparations are similar to those needed for xylocholine or bretylium. These substances are potent local anaesthetics (Boyd, Chang & Rand, 1960; Morales-Aguilera & Vaughan Williams, 1963) but in the cases of xylocholine (Bain, 1960), bretylium (Boyd & others, 1960) and guanethidine (Bein, 1960) there are powerful arguments for dissociating this property from adrenergic neuron blocking activity. No such evidence exists for propranolol and indeed the failure of a dose of 15 mg/kg intravenously to affect post-ganglionic stimulation of the cat nictitating membrane (Raper & Wale, 1969) argues strongly against the relevance of these observations *in vitro* to the situation *in vivo*.

The release of noradrenaline from isolated nerve granules is blocked by propranolol at 3×10^{-4}M (77·5 μg/ml) (Euler & Lishajko, 1966). Similarly, uptake of noradrenaline either by isolated nerve granules (Euler & Lishajko, 1966) or by isolated rabbit hearts (Foo, Jowett & Stafford, 1968) is also inhibited by similar concentrations of propranolol. In contrast, practolol had no effect in the present experiments or those of Foo & others (1966) and was found by Papp & Vaughan Williams (1969) to possess 1/100 the activity of propranolol as a local anaesthetic. Inhibition of noradrenaline uptake may not be irrelevant in man since the elevated excretion of noradrenaline during exposure to a sauna bath (Huikko, Jouppila & Kärki, 1966) is

enhanced by pretreatment with propranolol (10 mg) whilst elevation of plasma free fatty acids is inhibited (Arvela & Huikko, 1969). Furthermore, the potentiation of electrically stimulated contractions observed in the present experiments at lower concentrations of propranolol and the potentiation of exogenous noradrenaline would also be compatible with a decrease in the uptake of released noradrenaline.

The fact that the (+)-isomer of propranolol exerts a similar local anaesthetic (Barrett & Cullum, 1968) and adrenergic neuron blocking properties argues against the relevance of β-adrenoreceptor blocking activity in the present context. However, a non-specific axonal blockade could well account for the previously unexplained inhibition of vagal slowing following the sudden release of carotid occlusion after larger (3 mg/kg) doses of propranolol when smaller doses (0·25 mg/kg) are sufficient to inhibit the associated reflex tachycardia (Ledsome, Linden & Norman, 1965).

In conclusion it cannot be proved that the non-specific adrenergic neuron blocking activity of propranolol is not contributing to the hypotensive action of this drug in man but this effect could well augment the effects of β-blockade on cardiac output. The absence of any clear-cut orthostatic hypotension in patients receiving propranolol (Prichard & Gillam, 1969) does, however, militate against this speculation.

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The effect of acute adrenalectomy on the blood pressure responses to noradrenaline and to preganglionic nerve stimulation

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The quantitative contribution of the adrenal medulla to the pressor response resulting from stimulation of the entire preganglionic sympathetic spinal outflow of the rat, has been estimated. Adrenalectomy reduced the pressor responses not only to preganglionic nerve stimulation but also to injected noradrenaline. This reduction in cardiovascular sensitivity occurred within about 10 min of adrenalectomy and continued throughout the course of each experiment. Intramuscular injection of cortisone (20 mg/kg) restored cardiovascular sensitivity to injected noradrenaline thus allowing analysis of the adrenal medullary component of the overall response to spinal nerve stimulation.

The contribution of the adrenal medulla to the pressor response elicited by stimulation of the whole spinal sympathetic vasomotor outflow was considered by Gillespie & Muir (1967) to be small and variable. Since it was desired to use this preparation to examine ganglionic and adrenal medullary stimulants, an estimate of the contribution of the adrenal medulla to the total pressor response has been undertaken.

EXPERIMENTAL

C.S.E. male or female rats, 200–300 g, were pithed (Gillespie & Muir, 1967) under ether–halothane anaesthesia, and artificially respired using a Palmer miniature ideal pump at a stroke volume of 1 ml/100 g and at a rate of 50 strokes/min. Total bilateral adrenalectomy, via two lateral incisions was made using the same anaesthetic. "Sham adrenalectomized" rats were prepared by leaving the adrenal glands untouched after the incisions had been made.

The pithing rods, made from 16 gauge stainless steel wire, were dipped in Shellac varnish and, when dry, the area which was eventually to lie in the thoracico-lumbar region of the spinal cord was scraped clear of varnish. This length of rod had been previously determined in an animal of comparable size. An area of rod lying outside the body was also cleared of insulating varnish for attachment of an electrode. The indifferent electrode (a flattened serum needle) was placed subcutaneously in the dorsum.

Blood pressure was recorded from the left common carotid artery, using a Bell and Howell pressure transducer (Type 4-326-L212) and displayed on a Devices two channel pen recorder.

Heparin, 2000 units/kg was injected intravenously at the beginning of the experiment. Drugs were injected into the left femoral vein in a volume not exceeding 0·15 ml and were washed in with 0·2 ml saline.

Parameters of stimulation

Square wave pulses of 0·03 ms duration were delivered from a Multitone stimulator at a rate of 3 Hz for 15 s at intervals of 5 min. To eliminate any blood pressure change resulting from a change in heart rate, a stimulation strength of 20 V was used because, although of submaximal stimulation strength, previous examination using a heart rate recording system (Drew, Fozard & others, 1970) indicated that little or no change in heart rate occurred during stimulation at 20 V or less. Gillespie, MacLaren & Pollock (1969) have shown that heart rate and blood pressure changes can occur independently in this type of preparation.

The parameters used ensured that each pressor response showed no evidence of any residual effects from the preceding treatment.

Violent muscle contractions which occurred throughout the period of stimulation were prevented by the intravenous administration of 1 mg/kg (+)-tubocurarine.

Drugs

The drugs used were cortisone acetate (Cortistab—Boots Pure Drug Co. Ltd.), hydrocortisone sodium succinate (Glaxo), deoxycortone acetate (Organon), adrenaline acid tartrate (B.D.H.), tubocurarine chloride (Tubarine—Burroughs Wellcome & Co.) and noradrenaline acid tartrate (Hoechst). Apart from deoxycortone acetate, all drugs were injected in 0·9% sodium chloride solution, and doses calculated as the salt except in the cases of noradrenaline and adrenaline which were made up from stock solutions, stored in 0·01N hydrochloric acid, of 1 mg/ml calculated as the base.

Deoxycortone acetate was made up as 2 mg/ml suspension in propylene glycol.

RESULTS

Intact rats

Responses to injected noradrenaline

The characteristic response to an intravenous injection of noradrenaline, 500 ng/kg, was a rapid rise in blood pressure commencing 2 or 3 s after injection, and reaching a peak after about 5 s, whereupon a characteristic lowering of the diastolic pressure occurred. The diastolic pressure subsequently recovered over the next 2 or 3 s, and occasionally exceeded its initial peak (Fig. 1a).

The rises in both systolic and diastolic pressures were well maintained and did not start to wane until about 30 s after the injection.

Responses to preganglionic nerve stimulation

The typical pressor response evoked by stimulation of the entire spinal sympathetic preganglionic outflow consisted of a rapid rise in blood pressure lasting some 10–15 s (Fig. 2a). Occasionally this rise occurred in two distinct phases (Fig. 2b) and was seen as an initial phase, which reached a plateau about 5 s after stimulation had begun, and a secondary phase which followed after 2 or 3 s. After having attained its maximum, the response often began to decline before stimulation had ended. The decline of the overall pressor response after cessation of stimulation was variable; at stimulation voltages above 20 V a definite inflexion in the recovery phase occurred about 20–30 s after the end of stimulation, and coincided with the response described by Gillespie & Muir (1967) as the adrenal medullary component of the response.

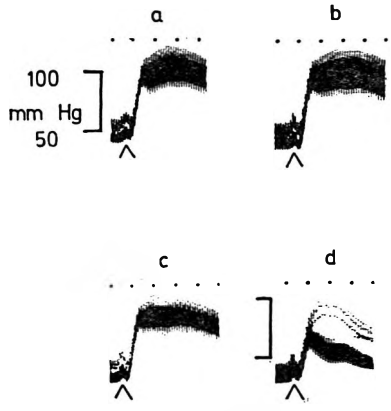

FIG. 1. The pressor responses evoked from an intravenous injection of noradrenaline 500 ng/kg i.v. a. The response in normal rats. b. The response in rats sham adrenalectomized 2 h previously. c. The response in rats adrenalectomized and treated with cortisone 20 mg/kg, i.m. 2 h previously. d. The response in rats adrenalectomized 2 h previously but with no replacement therapy. (NOTE different calibration). The slight response before each pressor response is the injection artifact and indicates the point of injection of noradrenaline. Time dots above traces are at 10 s intervals.

At 20 V stimulation strength, or less, this effect was less noticeable and appeared instead as a reduction in the overall rate of decline of the pressor response, frequently accompanied by an increase in pulse pressure (Fig. 2a, c).

The effect of acute adrenalectomy

The removal of, or ligating of the blood vessels of, the adrenal gland during the course of an experiment reduced the responses to preganglionic nerve stimulation. Such an effect was expected as a consequence of removal of the adrenal medulla, but the responsiveness to injected noradrenaline was also decreased. These effects

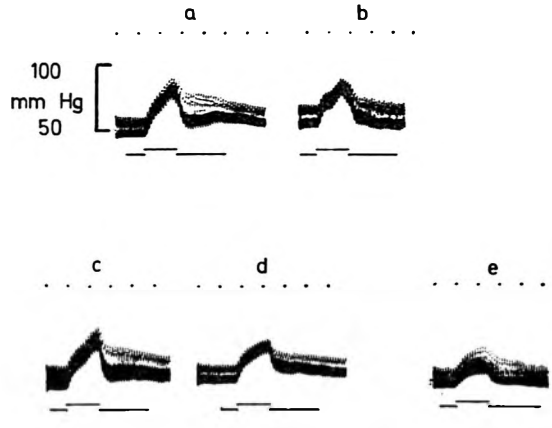

FIG. 2. The pressor responses evoked by stimulation of the entire spinal sympathetic preganglionic outflow at 20 V 0·03 ms pulse duration 3 Hz for 15 s. a + b. The response in normal rats. c. The response in rats, sham adrenalectomized 2 h previously. d. The response in rats adrenalectomized and treated with cortisone 20 mg/kg i.m. 2 h previously. e. The response in rats adrenalectomized 2 h previously, but with no replacement therapy. (NOTE: Different calibration.) Time bar below traces indicates period of stimulation. Time dots above traces are at 10 s intervals.

were first observable within 10 min of adrenalectomy and a continual decline in both types of response progressed throughout the course of the experiment. A progressive fall in the resting blood pressure usually accompanied these events, despite the fact that surgical procedures caused little or no haemorrhage.

The infusion of adrenaline

The infusion of adrenaline at the rate of 200–250 ng/kg min^{-1} over periods of up to 20 min raised the resting blood pressure by about 10 mm; pulse pressure and heart rate were also increased, but the responsiveness to either injected noradrenaline or preganglionic nerve stimulation was not restored to normal levels.

Adrenalectomized rats

Chronic experiments

In an attempt to overcome the difficulties arising from acute adrenalectomy, rats were adrenalectomized and chronically treated with a variety of replacement therapies. These involved saline substitution for drinking water and daily injections of deoxycortone acetate (1 mg/kg, s.c.). In some cases, hydrocortisone (2·5 mg/kg, s.c.) was also given. Such treatment lasted 3 to 4 weeks after adrenalectomy.

None of the replacement therapies restored cardiovascular sensitivity to catecholamines.

Hydrocortisone infusion

The infusion of hydrocortisone at 4–5 μg/kg min^{-1} for 15–20 min, into chronically adrenalectomized animals, maintained on deoxycortone acetate and saline only, failed to influence the decreased sensitivity to injected noradrenaline or nervous stimulation. However, it was observed that a single injection of cortisone, administered intramuscularly a short while before experimentation with these chronically adrenalectomized animals did result in some restoration of cardiovascular sensitivity towards catecholamines. Accordingly a more comprehensive study of the action of cortisone was undertaken.

The effects of cortisone administration

The effect of cortisone, administered at the time of adrenalectomy was examined 2–4 h after the operation. For comparison, adrenalectomized untreated rats and sham adrenalectomized rats were also examined. Table 1 compares the responsiveness to noradrenaline injection and preganglionic nerve stimulation in these preparations, and also in intact rats.

It is clear from these results that sham adrenalectomized and intact rats showed similar responsiveness to the test procedures, but that adrenalectomized rats differed, in that their resting blood pressure, after pithing, was 10–15 mm lower, and the pressor responses to preganglionic nerve stimulation and noradrenaline injection were markedly reduced.

Cortisone, 20 mg/kg intramuscularly, restored the resting blood pressure, after the animals had been pithed, to a level comparable with that observed in intact and sham adrenalectomized rats. The pressor responses to injected noradrenaline were also restored but the responses to preganglionic nerve stimulation was only partially restored.

Table 1. *The resting blood pressure, and the pressor responses to noradrenaline injection and total sympathetic preganglionic outflow stimulation in normal, sham adrenalectomized, adrenalectomized and adrenalectomized cortisone-treated pithed rats*

	Rise in systolic and diastolic pressures after i.v. injection of 500 ng/kg NA (in mm of Hg)	Diastolic pressure rise following stimulation of the total preganglionic sympathetic outflow (in mm of Hg)	Resting systolic and diastolic pressures after pithing (in mm of Hg)
Intact rats (11)	63·3 ± 2·8 55·0 ± 3·0	37·5 ± 2·5	66·3 ± 2·4 49·1 ± 2·0
Rats sham adrenalectomized, 2 h before experiment (10)	63·1 ± 1·9 49·6 ± 2·5	36·5 ± 2·5	65·6 ± 2·7 46·6 ± 1·8
Rats adrenalectomized and treated with cortisone 20 mg/kg i.m. 2 h before experiment (11)	62·1 ± 1·5 47·6 ± 2·8	27·9 ± 2·7*	67·7 ± 2·4 49·3 ± 1·6
Rats adrenalectomized 2 h before examination. No replacement therapy (10) ..	50·3 ± 2·4* 31·1 ± 1·0*	20·4 ± 2·5*	55·0 ± 0·7* 41·3 ± 1·1*

All values are given as the mean, ± the standard error of mean.
( ) denotes the number of animals in the group.
* $P < 0.05$ compared with normal rats.

Cortisone, 20 mg/kg intramuscularly, also prevented the occurrence of some gross changes in adrenalectomized animals. Shortly after adrenalectomy the animals' activity was reduced, respiration became slow and laboured and, characteristically, marked piloerection occurred.

Cortisone, 10 mg/kg intramuscularly, was only partially successful restoring cardiovascular sensitivity to catecholamines, and deoxycortone acetate, 5 mg/kg, by the same route was unsuccessful.

Apart from the quantitative aspects, described in Table 1, certain qualitative changes can also be discerned.

Noradrenaline injection

In adrenalectomized animals, the pressor response to injected noradrenaline was seen as a slowly occurring rise in pressure which was not well maintained, and declined immediately after having attained its peak. Since the diastolic pressure declined more rapidly than the systolic pressure a large increase in pulse pressure was apparent (Fig. 1d). Cortisone restored noradrenaline responsiveness to that observed in intact and sham adrenalectomized animals (Fig. 1a, b, c).

Preganglionic nerve stimulation

The responses to stimulation of the entire preganglionic sympathetic spinal outflow were similar in intact and sham adrenalectomized animals (Fig. 2a, b, c), but in adrenalectomized animals it was much reduced, and no secondary rise was ever apparent.

Systolic and diastolic pressures were equally increased (Fig. 2e). Cortisone pretreatment increased the size of the pressor response, but at no time did the secondary phase, apparent in intact and sham adrenalectomized preparations, return (Fig. 2d). Neither did the pressor response begin to decline until stimulation was ended, and the pulse pressure was generally reduced throughout the period of stimulation.

Consequently, since cortisone (20 mg/kg, i.m.) restored both the resting blood pressure and the responsiveness to noradrenaline injection, of adrenalectomized rats, to levels comparable with those observed in intact rats, it is suggested that the reduction in magnitude and change in shape of the response to preganglionic nerve stimulation was a consequence of the removal of the adrenal medullary component of the response. Thus it is estimated that the adrenal medullary component of the response, in intact animals, was about 24%.

Adrenal demedullation

Adrenal demedullation instead of total adrenalectomy, did not prevent the onset of reduced cardiovascular sensitivity when examined 2 h after demedullation. This was probably due to destruction of the functional integrity of the remaining adrenal cortex.

Fig. 3 indicates that 18 h after adrenalectomy there is a further reduction in cardiovascular sensitivity.

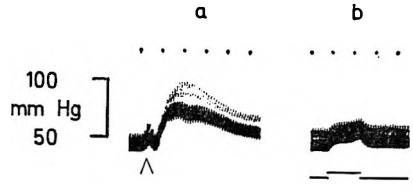

FIG. 3. The responses to injection of noradrenaline 500 ng/kg, i.v. (a) and to preganglionic stimulation at 20 V 0·03 ms pulse duration. 3 Hz for 15 s (b) in a rat adrenalectomized, and again no replacement therapy, 18 h previously. Time dots above tracing are at 10 s intervals.

DISCUSSION

Changes in cardiovascular function which occur as a result of adrenocortical insufficiency have been described by many authors (Ramey & Goldstein, 1957; Bush, 1962; Hofmann & Sobel, 1964). Typical symptoms are a reduction in pulse pressure, blood volume and peripheral resistance, and an inability of the heart to respond normally to changes in venous return or to increases in systemic arterial pressure. The condition is accompanied by a varying degree of hypotension.

Changes in cardiovascular sensitivity after adrenalectomy in the rat have been described by Zweifach, Shorr & Black (1953) using the rat mesoappendix preparation. This preparation showed a progressive decline in sensitivity to repeated injections of noradrenaline.

Imms & Jones (1968) have observed a decreased arterial pressure and decreased responsiveness to catecholamines in adrenalectomized rats, whilst Cartoni & Carpi (1968) demonstrated reduced responsiveness to endogenously released catecholamines after nerve stimulation.

These consequences of adrenalectomy probably account for a number of observations reported in this present work, namely:

(1) The inability of adrenalectomized rats to maintain the diastolic pressure rise following injection of noradrenaline.

(2) The failure of the diastolic pressure rise to exceed that of the systolic pressure, during preganglionic nerve stimulation in adrenalectomized animals only.

(3) The lowered resting systolic and diastolic pressures observed in adrenalectomized animals.

Furthermore, the piloerection observed in adrenalectomized rats probably reflects the reflexly induced hyperactivity of the sympathetic nervous system which occurs after adrenalectomy (Imms & Jones, 1968).

Carpi & Cartoni (1968) and Cartoni & Carpi (1968) have demonstrated that replacement therapy with both mineralocorticoids and glucocorticoids is necessary for the complete restoration of cardiovascular sensitivity. As in our results, they noted that deoxycortone acetate and saline administration were insufficient to maintain reactivity. That additional chronic (and acute) administration of hydrocortisone failed to restore sensitivity to catecholamines is in agreement with the findings of Rosenfeld, Sevy & Ohler (1959) and Ross (1961), who have shown that even large doses exert no effect on catecholamine sensitivity *in vivo*.

The ineffectiveness of adrenaline infusion to restore cardiovascular sensitivity indicates that a reduction in the background circulatory level of adrenal medullary hormones is not responsible for the changes following adrenalectomy, as reported by Harakal, Sevy & others (1968).

Acute administration of deoxycortone acetate was also without effect as observed by Ramey, Goldstein & Levine (1951).

However, acute administration of cortisone (Fritz & Levine, 1951; Zweifach, Shorr & Black, 1953) or corticosterone (Imms & Jones, 1967) proved useful in restoring cardiovascular responsiveness. The present work and that of Imms & Jones suggests that acute administration of corticosteroids of mixed activity may reverse the more immediate effects of adrenalectomy and that the more advanced changes which occur some time later may account for the conflict with the effects of chronic treatment demonstrated by Carpi & Cartoni. This may be a consequence of changes in ionic balance which occur, but no determination of these factors has been attempted in the experiments reported in this paper.

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The isolated perfused heart preparation: two suggested improvements

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A new method of recording the contractions of the isolated perfused heart is described. By "transverse" recording, a signal of simple waveform is obtained from the right ventricle at constant diastolic tension. Changes in heart rate and force of contraction of the rat isolated perfused heart caused by (−)-noradrenaline were measured. Of the three methods of assessing the response: (1) the increment as a percentage of the control record, (2) the increment in absolute units and (3) the increment as a percentage of the maximum, the latter has most pharmacological meaning.

The isolated heart preparation, perfused by the Langendorf (1895) technique, is widely used to assess the activity of drugs which affect myocardial contractility and heart rate.

The method of making the record

The usual method of recording cardiac contractions uses a small metal clip attached to the apex of the heart. The rhythmical contractions are transmitted by a length of thread to either a spring loaded writing lever or a force displacement transducer ("longitudinal" recording Fig. 1a). While attempting to investigate the action of sympathomimetic drugs on the rat heart by this method, using a transducer and pen recorder, certain disadvantages became apparent:

(*a*) By virtue of the positioning of the clip, the signal produced is composed of the contractions of the left and right ventricles.

(*b*) A rotational component of the heart's contraction is very obvious in the freely

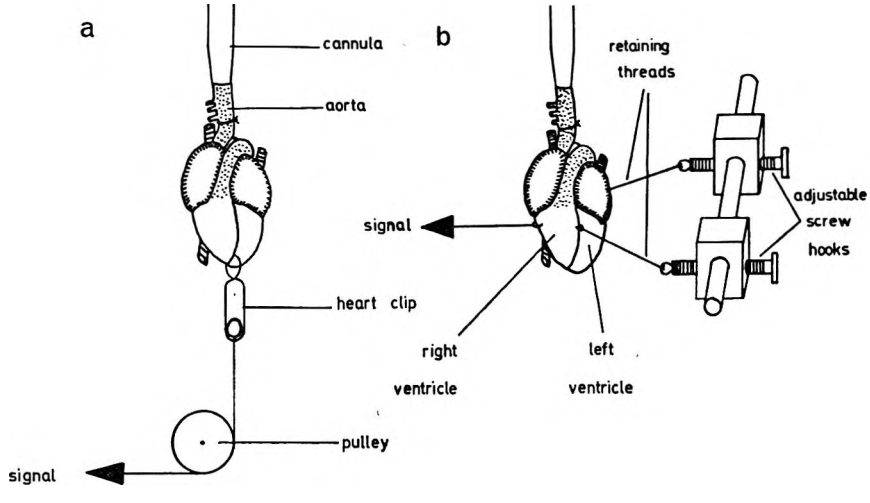

FIG. 1. a. "Longitudinal," b, "transverse" method of recording the contractions of the isolated perfused heart.

suspended preparation and this is added to the already compound signal from the heart clip.

(*c*) Instantaneous ratemeters for measuring heart rate and operational amplifiers for recording the rate of change of myocardial tension will only function accurately if the input signal has a *simple* waveform.

The problem of obtaining a homogenous signal from the contracting heart was overcome in the following manner. The heart was rapidly removed and suspended from a fine polyethylene cannula inserted into the aorta. This delivered warmed (37°), oxygenated (gassed with 5% carbon dioxide in oxygen) Krebs solution to the coronary circulation at a constant flow rate of 8 ml/min. Before securing the heart to the cannula it was rotated so that the right ventricle was facing an Ether 2 oz dynamometer (UF1). A stitch of terylene thread was inserted in the ventricular *septal* tissue on both the anterior and posterior surfaces of the heart approximately midway between the base and apex, and securely tied (Fig. 1b). Occlusion of any coronary vessels was carefully avoided. Each of the two threads was then tied to an adjustable screw hook mounted on a horizontal rod about 3 in from the heart and level with it so that the threads, when tightened, were horizontal and prevented any rotational movement of the contracting heart. A third stitch of terylene thread was inserted in the centre of the surface of the right ventricle in the same plane as the two retaining threads and passed to the dynamometer. This method of recording from the isolated heart was termed "transverse" recording. Alteration of the distance of the dynamometer from the heart by manipulation of a rackwork "X" block allowed fine control of the diastolic tension to which the right ventricle was subjected. A comparison of the records obtained with these two systems is shown in Fig. 2. The two records were obtained from the same heart at the same diastolic

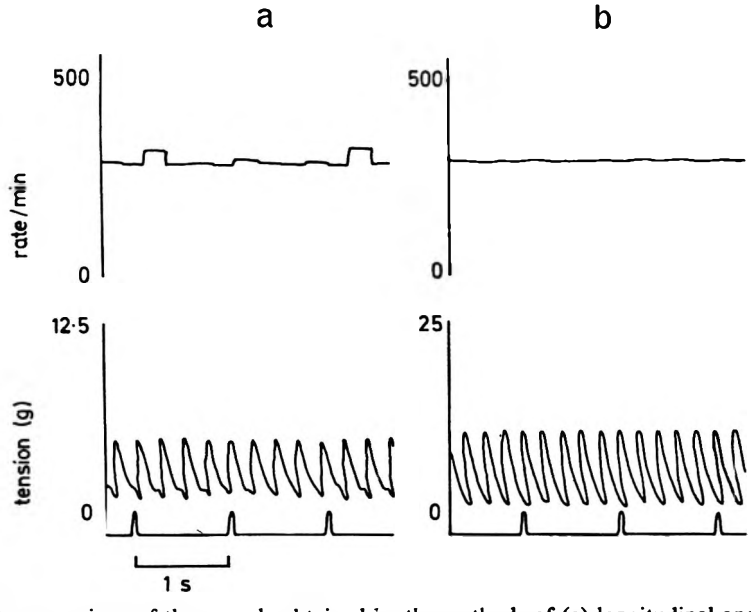

FIG. 2. A comparison of the records obtained by the methods of (a) longitudinal and (b) transverse recording of the contractions of the same rat isolated perfused heart. The diastolic tension was 1·5 g.

tension; a pure waveform is achieved by "transverse" recording with approximately twice the amplitude of the "longitudinal" record. Such a wave form has been successfully used as the signal for operational amplifiers measuring the rate of change of myocardial tension (differentiated record), measuring the area under the curve of the cardiac tension cycle over a predetermined time (integrated record), and also for instantaneous ratemeters.

Preliminary experiments using different diastolic tensions applied to the right ventricle revealed a very steep linear relation between diastolic tension and both the control force of contraction and the increment in force caused by a dose of noradrenaline. Since the diastolic tension alters spontaneously during the course of an experiment (usually it declines) it was readjusted to 1·5 g one min before each drug injection.

The method of presenting the results

The positive chronotropic and inotropic responses of the isolated heart preparation to injections of sympathomimetic drugs into the coronary circulation are expressed in one of two ways when attempting to assess the response to the drug quantitatively. Either the increment in rate or force of contraction caused by the drug is expressed as a proportion (%) of the magnitude of the parameter recorded immediately before drug injection (the commonest method, usually referred to as "percentage of control") or alternatively the size of this increment is given in absolute units. These two methods of presentation of results were compared on six rat isolated perfused hearts as follows. Doses of noradrenaline of 0·2, 1·0, 5, 50, 500, 1200 and 2400 ng were

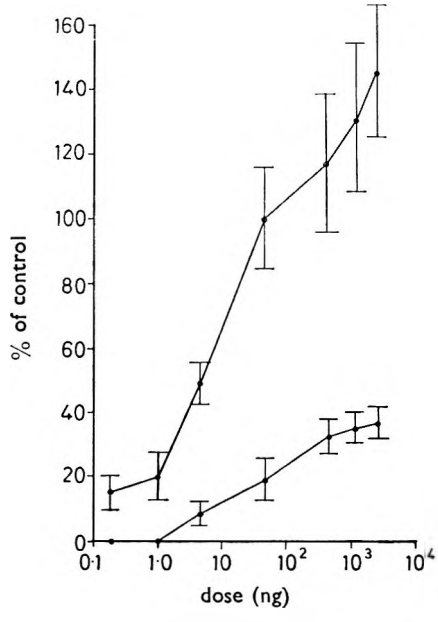

FIG. 3. Log dose response curves to (—)-noradrenaline on the rat isolated perfused heart preparation at a diastolic tension of 1·5 g. Heart rate = —●—●—. Right ventricular systolic tension = —○—○—. The response plotted is the maximum increment caused by noradrenaline expressed as a percentage of the heart rate or right ventricular systolic tension immediately preceding the injection. Each point is the mean of six experiments ± standard error.

injected in a volume of 0·05 ml into the aortic cannula of each rat heart, prepared for "transverse" recording. The dose sequence was randomized with a 10 min interval between doses.

When the results obtained with noradrenaline are presented as a percentage of the control value, the mean log dose response curves appear as in Fig. 3. Inspection of the records showed that the absence of a plateau in the tension log dose response curve is due not to an increased increment caused by the higher doses of noradrenaline but to an irreversible decline in the control tension record following these higher doses. The standard errors of the mean responses for most doses are large when presented in this way since they are compounded of three sources of variance: (i) that between hearts; (ii) that of regression of the increment of tension on the log dose noradrenaline in each heart; (iii) that of the magnitude of the control record which irreversibly declines after high doses and even in the untreated preparation is prone to spontaneous variation.

Since at constant diastolic tension the increment in tension produced by any dose of noradrenaline appeared to be independent of the control value there seemed no merit in including the size of this control value in the response presented.

When the results obtained with noradrenaline are presented as the increment in absolute units for the rate (beats/min) and force of contraction (tension in g), the mean log dose response curves for both now show a plateau still with large standard errors about the mean for any one dose (Fig. 4). When a constant dose of noradrenaline (ED50) was administered at 10 min intervals to one heart over a period

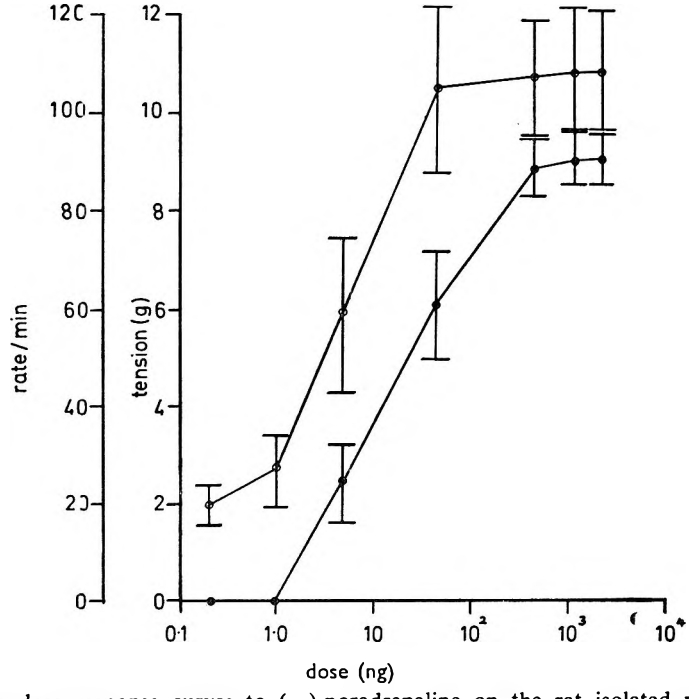

FIG. 4. Log dose response curves to (—)-noradrenaline on the rat isolated perfused heart preparation at a diastolic tension of 1·5 g. Heart rate = —●—●—. Right ventricular systolic tension = —○—○—. The response plotted is the maximum increment caused by noradrenaline in absolute units. Each point is the mean of six experiments ± standard error.

of 2 h the increment remained constant, so the large standard errors are indicative of the operation of two compounded sources of variance (i and ii above).

With other isolated tissues there are good theoretical reasons for the common practice of relating the response obtained with a dose of drug to the maximum response which may be achieved by the drug in that tissue. When the increments to noradrenaline are expressed in this way, the mean log dose response curves for each parameter (Fig. 5) have reduced variability compared with the previous method. It is felt that this represents the method of choice, only the variability between hearts remaining. The slope and position of the mean log dose response curves are not

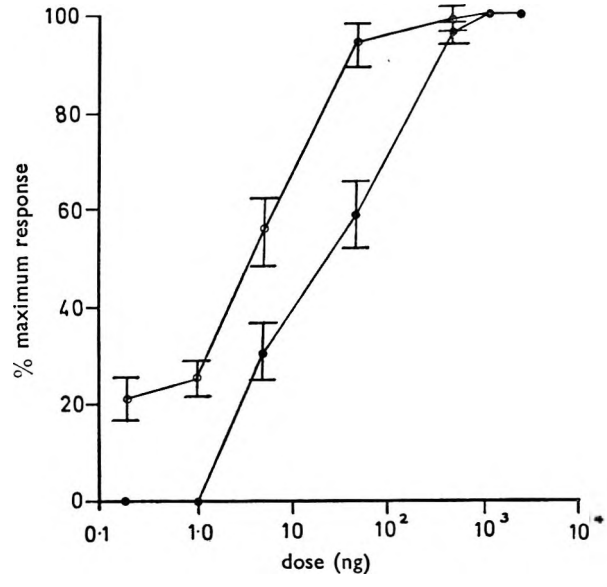

FIG. 5. Log dose response curves to (—)-noradrenaline on the rat isolated perfused heart preparation at a diastolic tension of 1·5 g. Heart rate = —●—●—. Right ventricular systolic tension = —○—○—. The response plotted is the maximum increment caused by noradrenaline expressed as a percentage of the increment caused by a dose of 2·4 μg noradrenaline. Each point is the mean of six experiments ± standard error.

significantly different at diastolic tensions of 1·5, 2·5, 3·5 and 4·5 g when the increment is calculated as a percentage of the maximum response. Thus, as long as the diastolic tension remains constant throughout a dose sequence, log dose response curves are comparable. An obvious further improvement to recording responses of the preparation by "transverse" recording with manual re-adjustment of the diastolic tension would be a servo-mechanism whereby the diastolic tension could be maintained at a constant preset value to minimise the errors resulting from the decline which is observed.

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Some effects of experimentally-produced cigarette smoke on the growth, vitamin C metabolism and organ weights of guinea-pigs

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Guinea-pigs receiving a controlled dietary intake of L-xyloascorbic acid (ascorbic acid, vitamin C) inhaled experimentally produced cigarette smoke for periods of up to 20 min each day. Growth rate was significantly depressed by the smoke treatment, an effect at least in part attributable to a reduction in food intake. Growth of individual organs was not depressed to the same extent as that of the body as a whole. The lungs of the animals receiving smoke were significantly heavier than those of control animals ($P < 0.05$). The concentration of ascorbic acid in the adrenal glands was significantly lower in the animals receiving smoke than in the controls ($P < 0.01$). The smoke-induced depression of the adrenal gland ascorbic acid was apparent after 4 days; after 18 days marked adrenal hypertrophy accompanied the lowered ascorbic acid levels.

Lupu, Velican & Mihaescu (1954) found that exposure of guinea-pigs and rabbits to cigarette smoke resulted in a fall in the ascorbic acid concentration in the adrenal glands of both species. We have not found any reports of smoke-induced changes in the ascorbic acid content of any other organs. There is evidence that in man smokers have lowered blood levels of vitamin C (Venelut, 1954; Calder, Curtis & Fore, 1963).

The experiments now described were designed to cast further light on this relation. Guinea-pigs were used since their complete dependence upon dietary vitamin C enables tissue levels to be established at pre-determined values. The animals were exposed to controlled amounts of experimentally produced cigarette smoke and its effects on the growth rate and on the ascorbic acid content and weight of certain organs were recorded.

EXPERIMENTAL

Animals and diet

Male albino guinea-pigs, initial weight 250–300 g, were used. The diet had the following composition (g); ground oats 37, wheat bran 35, dried skim milk (1% fat) 10, dried full-cream milk (26·5% fat) 10, dried yeast 6·5, salt mixture 1·0, magnesium oxide 0·5. The dried milks were from Unigate (Milk Products) Ltd., the dried yeast from Distillers Co. (Yeast) Ltd. and the salt mixture from Glaxo Laboratories Ltd. Before incorporating the milk powders into the diet they were heated for 24 h at 110° to destroy any residual ascorbic acid. Each animal received a weekly supplement of 0·05 ml of menaphthone in arachis oil (5% w/v), 0·05 ml of DL-α-tocopherol acetate in arachis oil (5% w/v) and 0·05 ml of cod liver oil. Previous work had already indicated that this diet, when supplemented with ascorbic acid, supported normal

growth of guinea-pigs; without the ascorbic acid the guinea-pigs developed scurvy (Hughes & Hurley, 1969). A daily dose of ascorbic acid was given orally on a body-weight basis to all animals.

The guinea-pigs were housed in individual galvanized zinc cages with removable 7/10-inch mesh bottoms. They were allowed to eat the diet freely. When measurements of food intake were required, 50 g of diet was given daily to each animal and the amount left uneaten 24 h later measured.

Organ weights and ascorbic acid analysis

At the end of each experiment the animals were killed by stunning and decapitation. The appropriate organs were rapidly dissected, dried once between filter paper and weighed. An extract for ascorbic acid determination was prepared by grinding the weighed organ with sand and 6% metaphosphoric acid; the reduction of a standard acid solution of phenol-indo-2,6-dichlorophenol by a portion of the extract was measured photometrically (Hughes, 1956).

Production of the cigarette smoke and its presentation to the guinea-pigs

The cigarettes were smoked in a standard smoking machine (R. W. Mason, Clevedon, Somerset). Each cigarette was drawn on once a minute by the machine for 2 s at a suction pressure of 350 mm of water. These values are based on measurements quoted for human smokers (Fabricant, 1946; Shepherd, 1951; Greenberg, Lester & Haggard, 1925; Hilding, 1956; van Proosdy, 1960). The cigarettes were burnt to a stub length of 20 mm (Doll, Hill & others, 1959; Korteweg, 1959).

The smoke entered an adaptor (manufactured and fitted by East & Co. Ltd., Cowley, Oxford) where it was mixed with air to give a composition similar to that known to be present in the lungs of smokers whilst smoking (about 500 ml of air containing 34 ml of smoke). The mixture was propelled along a Perspex tube containing a row of ports along each side fitted with rubber adaptors to accommodate heads of different sizes. The guinea-pigs were placed in wood–metal restrainers fitted with adjustable metal positioning rods and moved into position so that the head, up to, but excluding, the eyes, projected through the port into the tube. They appeared normal when replaced in their cages after being subjected to the smoke. The control animals were similarly treated but were not exposed to smoke. This procedure produces a situation where, instead of receiving a smoke–air mixture once a minute, the lungs are continuously filled with it. Methodological limitations make it difficult to simulate more closely the human situation.

Plan of experiments

Expt 1. Two groups of 12 animals were used. One was a control group and the other was exposed to the smoke–air mixture for a single period of 10 min daily, for 51 days, the animals being weighed daily. The animals were then killed, the adrenals, heart, lungs, kidney, brain, liver and testes removed and weighed and the ascorbic acid content of the adrenals, testes and brain determined.

Expt 2. Two groups of 15 animals were used. The experiment continued for 29 days and the animals were exposed to the smoke–air mixture for two periods of 10 min daily (at 09.30 and at 21.30 h). Food intake was measured and the ascorbic acid content of the liver, testes and adrenal glands determined.

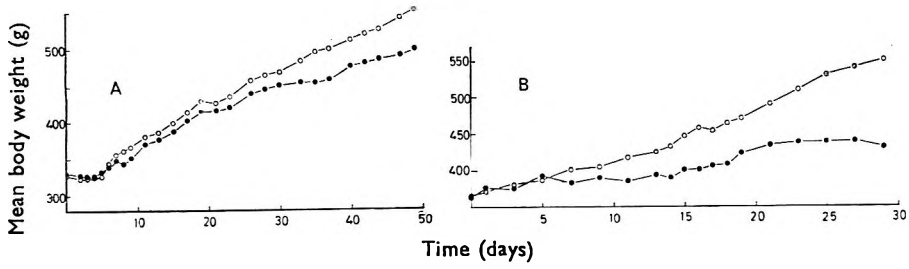

FIG. 1. Effect of smoke-air mixture [either one daily exposure (A, 12 animals/group) or two (B, 15 animals/group), each of 10 min] on growth of guinea-pigs. ○—○, control group; ●—●, smokers. Exposure to the smoke commenced on the fifth day.

Expt 3. Two groups of eight animals were used. The experimental conditions were as in Expt 2 but the experiment was terminated after 18 days. The weights and ascorbic acid contents of the adrenal glands and spleen were determined.

Expt 4. This was designed to determine how rapidly the smoking effects appeared. Two groups of animals were treated as in Expt 3 for 4 days.

RESULTS

These are given in Fig. 1 and in Table 1. Analysis of variance tests for significance were applied and a probability of $\leqslant 0{\cdot}05$ accepted as revealing a significant difference. Guinea-pigs exposed to the cigarette smoke for a single period of 10 min daily (Expt 1) had a lower growth rate than the corresponding control group (Fig. 1) the mean body-weights of the two groups being significantly different ($P \leqslant 0{\cdot}05$) from the 35th day of treatment. The ascorbic acid levels in the organs

Table 1. *Effect of smoke treatment (two 10-min exposures daily) on weight and ascorbic acid content of guinea-pig organs.* The daily intake of ascorbic acid was 0·5 mg/100 g. (Mean values with their standard errors)

Period of smoke treatment (days)	Guinea-pigs per group	Weight (g)	Weight as % of body-weight	Ascorbic acid (mg/100 g)
Adrenal glands				
C 4 (Expt 4)	8	0·127 ± 0·007	0·039 ± 0·002	30·5 ± 0·76†
S 4 (Expt 4)	8	0·129 ± 0·016	0·039 ± 0·005	21·9 ± 0·58
C 18 (Expt 3)	8	0·204 ± 0·004*	0·047 ± 0·009*	31·7 ± 0·76†
S 18 (Expt 3)	8	0·271 ± 0·007	0·067 ± 0·003	19·8 ± 0·88
C 29 (Expt 2)	15	0·236 ± 0·010	0·040 ± 0·002*	31·1 ± 1·5†
S 29 (Expt 2)	15	0·270 ± 0·011	0·062 ± 0·006	22·0 ± 2·2
Spleen				
C 4 (Expt 4)	8	0·409 ± 0·004	0·128 ± 0·001	8·6 ± 0·34
S 4 (Expt 4)	8	0·391 ± 0·005	0·169 ± 0·013	7·9 ± 0·24
C 18 (Expt 3)	8	0·300 ± 0·020	0·084 ± 0·004	8·1 ± 0·24
S 18 (Expt 3)	8	0·350 ± 0·015	0·085 ± 0·001	6·5 ± 0·24
Testes				
C 29 (Expt 2)	15	2·70 ± 0·08	0·46 ± 0·02	8·88 ± 0·16
S 29 (Expt 2)	15	1·96 ± 0·18	0·45 ± 0·034	8·09 ± 0·05

* Difference of means for controls (C) and smoke-treated animals (S) significant at 5% level.
† Difference of means for controls and smoke-treated animals significant at 1% level.

examined (adrenals, testes, brain) were not significantly different. Of the organs examined only with the lung was there a significant difference between the absolute weights. The mean absolute weight for the lungs of the controls was 3·16 g $\pm$ 0·11 (0·55 $\pm$0·01 as % of body-weight) and for the smoke-treated animals 3·64 $\pm$ 0·19 (0·71 $\pm$ 0·02 as % of body-weight) ($P < 0·05$).

In Expts 2–4 the animals were subjected to two 10 min periods of smoke treatment daily. This intensification of the treatment produced a still greater divergence in the growth curves of the two groups, the difference in mean body-weight being significant ($P < 0·05$) from the 11th day of treatment (Fig. 1B). The adrenal ascorbic acid was in this experiment significantly lower in the animals receiving smoke than in the controls ($P < 0·01$; Table 1). During the period when food intake measurements were made (days 12–28) the controls ate significantly more food on a body-weight basis than the smoke-treated animals ($P < 0·01$).

Experiments 3 and 4 were short-term (18 and 4 days respectively). In the 18-day experiment exposure to the smoke produced both adrenal hypertrophy and a lowered adrenal ascorbic acid level (Table 1). In the 4-day experiment there was no hypertrophy of the adrenal glands although their ascorbic acid level was depressed ($P < 0·01$; Table 1). There was no significant change in the ascorbic acid content of the spleen (Expts 3 and 4) or of the testes (Expt 2) of the smoke-treated animals (Table 1).

DISCUSSION

Evidence suggestive of a possible relation in man between smoking and ascorbic acid derives primarily from measurements of blood levels of the vitamin (Venelut, 1954; Calder & others, 1963; Brook & Grimshaw, 1968). It is generally accepted that in man the level of ascorbic acid in the leucocytes (and to a less extent, in the plasma) provides a useful index to the ascorbic acid status of the body as a whole (Vilter, 1967). In guinea-pigs, however, this is not so, particularly at the sub-optimal dietary levels we used (Hughes & Jones, 1970). On the other hand, the ascorbic acid content of organs such as the adrenal glands and the spleen appears to reflect dietary intake over a very wide range and its determination in such organs presents none of the difficulties associated with its determination in leucocytes. In most experiments, the ascorbic acid content of the adrenals and spleen was, therefore, assumed to reflect the ascorbic acid status of the animal.

The guinea-pigs received a controlled daily intake of ascorbic acid of 0·3 mg/100 g body-weight (Expt 1) or 0·5 mg/100 g body-weight (Expts 2–4). This is sufficient to maintain growth and development but is well below the intake necessary to produce tissue saturation (Evans & Hughes, 1963). Restriction of the ascorbic acid intake to these pre-determined levels meant that any smoke-induced changes in the vitamin C economy of the body as a whole would be reflected as changes in the tissue levels.

The most striking result was a fall in the ascorbic acid concentration of the adrenal glands of the animals receiving smoke. Stressing agents such as low temperature, are known to produce a fall in adrenal ascorbic acid and Larson, Haag & Silvette (1961) suggested that smoking should be considered a stressing agent. This is now reinforced by two further findings; (*a*) the animals receiving smoke over the prolonged period had hypertrophied adrenal glands—this too being a condition characteristic of stressed animals, and (*b*) none of the other organs examined displayed

a fall in ascorbic acid. When the vitamin C status of the animal as a whole changes any change in the adrenal glands is paralleled by changes in other organs such as the spleen (Hughes & Jones, 1970).

It is unlikely that the lowered growth rate of the smoke-treated guinea-pigs is a consequence of changed tissue ascorbic acid levels. A large reduction of tissue ascorbic acid is necessary to produce the depressed growth rate characteristic of hypovitaminosis C (Evans & Hughes, 1963). Furthermore, the growth depression observed with the animals receiving smoke is attributable (at least in part) to a reduced food intake. Thus in Expt 2 from day 12 to day 28 of the experimental period, on a body-weight basis the food intakes of the controls were significantly higher ($P < 0{\cdot}01$) than those of the smoke-treated animals. This inanition presumably resulted from a direct effect of the smoke on the food intake control mechanism(s) although during the initial week of the experiment a depression of growth was recorded without any reduction of food intake (Fig. 1B).

Although the animals receiving smoke appeared to have larger organs on a body-weight basis, only the lungs (in absolute terms as well as on a body-weight basis) were significantly greater than in the controls. This would appear to indicate a true smoke-induced hypertrophy of the lungs in guinea-pigs, a phenomenon that does not appear to have been previously reported.

Acknowledgements

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The interaction of ethanol and amphetamine metabolism

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Ethanol, 1, 3, and 5 g/kg, depresses the hydroxylation of amphetamine by the rat *in vivo*. At 5 g/kg, ethanol does not affect the hydroxylation of acetanilide or biphenyl *in vivo*. Amphetamine hydroxylation is unaffected by phenobarbitone or benzo[a]pyrene pretreatment but is depressed by pretreatment with 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525-A), 2,4-dichloro-6-phenylphenoxyethylamine (DPEA), and 2,4-dichloro-6-phenylphenoxy-*NN*-diethylethylamine (Lilly 18947).

We have reported previously that ethanol treatment markedly inhibits the hydroxylation of amphetamine by the rat *in vivo* (Creaven & Barbee, 1969). We have now made further investigations of this and other aspects of amphetamine hydroxylation in an attempt to determine the mechanism of this inhibition by ethanol and to elucidate the differences between amphetamine hydroxylation and that of other drugs.

EXPERIMENTAL

Materials and methods

Male Sprague-Dawley rats, 100 to 150 g, received intraperitoneal injections of (±)-[2-^{14}C]amphetamine sulphate 5 mg/kg (1·6 mCi/mM), acetanilide (250 mg/kg in isotonic saline) or biphenyl (200 mg/kg in arachis oil). The animals were placed in metabolism cages and urine samples collected in flasks at 0 to 2°. Urinary pH was recorded and the urine frozen.

Animals were pretreated with 1, 3, or 5 g/kg ethanol by stomach tube as a 25% solution (v/v) in water 30 min before the administration of amphetamine. Control animals received an equal volume of saline.

Benzo[a]pyrene (2 mg/kg) and phenobarbitone (80 mg/kg) were administered intraperitoneally daily for three days; amphetamine was injected 24 h after the final dose. 2-Diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525-A), 2,4-dichloro-6-phenylphenoxyethylamine hydrochloride (DPEA), and 2,4-dichloro-6-phenylphenoxy-*NN*-diethylethylamine hydrobromide (Lilly 18947), 35 mg/kg, were injected intraperitoneally 45 min before the administration of amphetamine.

Pyrazole (100 mg/kg in isotonic saline) and disulfiram (100 mg/kg in arachis oil) were injected intraperitoneally 15 and 30 min, respectively, before oral administration of 3 g/kg of ethanol. Amphetamine was injected 30 min after ethanol administration.

Metabolites were identified by two-dimensional paper chromatography of urine to which authentic amphetamine and *p*-hydroxyamphetamine had been added. Chromatograms were developed in 1-butanol–acetic acid–water (4:1:1 v/v) followed by 2-propanol–ammonia–water (8:1:1 v/v) (Asatoor, Galman & others, 1965).

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After exposure to Kodak no-screen X-ray film NS 54-T, the films were developed and the chromatograms sprayed with diazotized *p*-nitroaniline. The conjugated *p*-hydroxyamphetamine was identified after incubation overnight at 37° with β-glucuronidase at pH 5·2.

For quantitation of metabolites, aliquots of urine were chromatographed on Whatman 3 MM or Whatman No. 1 paper in 1-butanol–acetic acid–water (4 : 1 : 1 v/v) or formic acid–isoamyl alcohol–t-amyl alcohol–water (2 : 5 : 5 : 10 v/v) (Alleva, 1963; Ellison, Gutzait & Van Loon, 1966).

Strips were dried and cut in ½ inch segments beginning ½ inch behind the origin. Each segment was placed in a liquid scintillation vial in 15 ml of liquid phosphor and counted in a Model 3375 Packard Tri-Carb Liquid Scintillation Spectrometer. Amphetamine metabolites are reported as percentages of excreted radioactivity.

Blood alcohol levels were sampled in amphetamine treated rats 12½ h after 5 g/kg of ethanol and measured by the method of Roach & Creaven (1968). *p*-Aminophenol was determined by the method of Brodie & Axelrod (1948); 4-hydroxybiphenyl by the method of Creaven, Parke & Williams (1965). These metabolites are reported as percentages of the dose.

RESULTS

The excretion of unchanged amphetamine is increased and that of *p*-hydroxyamphetamine is decreased by doses of 1, 3, and 5 g/kg of ethanol (Table 1). At the

Table 1. *Effect of ethanol pretreatment on the percentage of* p-*hydroxyamphetamine excreted in rat urine during various time periods after* (±)-[2-^{14}C]*amphetamine.*

Pretreatment	0–3 h	3–6 h	6–12 h	12–24 h
Saline (4)	44·7 ± 3·0	72·3 ± 3·4	74·4 ± 5·2	79·5 ± 6·2
Ethanol 1 g/kg (3)	23·2 ± 10·7‡	43·1 (1)‡	66·4 ± 9·1	66·7 ± 2·9‡
Ethanol 3 g/kg (5)	14·8 ± 7·1*	27·0 ± 5·8*	47·1 ± 9·2*	62·4 ± 8·2†
Ethanol 5 g/kg (2)	11·2 ± 2·3*		40·0 ± 0·5*	50·8 ± 6·9†
Ethanol 5 g/kg 14 h before amphetamine (4)	38·6 ± 5·5		72·2 ± 5·2	72·8 ± 9·7

Numbers of animals are shown in parentheses. Values are reported as mean ± s.d. * $P < 0·01$. † $P < 0·02$. ‡ $P < 0·05$.

beginning of the 12 to 24 h period, maximum blood ethanol levels after 5 g/kg of ethanol measure 1·2 mg/100 ml. Excretion of *p*-hydroxyamphetamine between 12 and 24 h after dosage remains significantly lower than the controls for all ethanol doses. However, hydroxylation of amphetamine injected 14 h after ethanol (5 g/kg) is not significantly different from controls (Table 1).

Combined pretreatment with ethanol and pyrazole produces a more profound effect on amphetamine metabolism which does not return to normal values by 24 h (Table 2). However, some inhibition of amphetamine hydroxylation occurs with pyrazole alone. Pretreatment with disulfiram does not increase the effect of ethanol on amphetamine metabolism although disulfiram without ethanol has an effect similar in magnitude to that of pyrazole alone (Table 2).

The hydroxylation of amphetamine is markedly inhibited by pretreatment with

Table 2. *Effect of pretreatment with ethanol in combination with pyrazole or disulfiram on the percentage of* p-*hydroxyamphetamine excreted in rat urine during various time periods after* (±)-[2-^{14}C]*amphetamine.*

Pretreatment	0–3 h	3–6 h	6–12 h	12–24 h
Ethanol (5)	14·8 ± 7·1	27·0 ± 5·8	47·1 ± 9·2	62·4 ± 8·2
Pyrazole and ethanol (5)	7·1 ± 0·8	16·0 ± 4·8	12·7 ± 6·5	15·5 ± 6·8
Disulfiram and ethanol (4)	7·1 ± 3·8	29·1 ± 7·7	45·6 ± 3·8	60·4 ± 4·0
Pyrazole (5)	20·3 ± 5·7	38·3 ± 6·4	40·3 ± 3·6	46·3 ± 5·3
Disulfiram (3)	24·8 ± 9·9	36·3 ± 6·5	55·3 ± 5·5	65·4 ± 5·0

Numbers of animals are shown in parentheses. Values are reported as mean ± s.d. Doses were: ethanol, 3 g/kg, pyrazole and disulfiram, 100 mg/kg.

SKF 525-A, DPEA, and Lilly 18947 (Table 3), but is unaffected by pretreatment for three days with phenobarbitone or benzo[a]pyrene (Table 3).

Pretreatment with ethanol (5 g/kg) has no effect on the amount of 4-hydroxyacetanilide or 4-hydroxybiphenyl excreted in 24 h after dosage with acetanilide or biphenyl. The amount of acetanilide hydroxylated is 53·8 ± 3·0% after ethanol; 55·9 ± 5·4% after saline. Biphenyl hydroxylation is 16·1 ± 2·3% after ethanol; 22·6 ± 4·9% after saline.

DISCUSSION

The results show that ethanol in doses of 1, 3, and 5 g/kg produces a marked depression of hydroxylation of the aromatic ring of amphetamine, the major metabolic pathway of this compound in the rat (Axelrod, 1954). The effect is most marked in the period immediately after ethanol treatment but can be seen 12 to 24 h after dosage. When amphetamine is injected 14 h after a dose of 5 g/kg of ethanol, no inhibition in *p*-hydroxylation is seen.

Pyrazole, an inhibitor of alcohol dehydrogenase (Lester, Keokosky & Felzenberg, 1968; Goldberg & Rydberg, 1969) greatly enhances the inhibition of amphetamine hydroxylation by ethanol. Disulfiram, an inhibitor of aldehyde dehydrogenase (Graham, 1951) does not enhance this inhibition. These results indicate that the inhibition of amphetamine metabolism is probably mediated through ethanol itself rather than by the metabolism of ethanol or by acetaldehyde. Pretreatment with pyrazole or disulfiram also depresses amphetamine hydroxylation, but to a lesser extent than when combined with ethanol. The reason for the inhibition of amphetamine metabolism by these agents is not presently known.

Table 3. *Effect of some inhibitors and inducers of liver microsomal oxidation on* p-*hydroxyamphetamine excretion in rat urine.* Urine was collected for 24 h.

Compound	*p*-Hydroxyamphetamine (% ± s.d.)	*P*
Saline (6)	65·7 ± 2·6	
DPEA (4)	11·6 ± 2·2	<0·01
SKF 525-A (4)	6·5 ± 0·8	<0·01
Lilly 18947 (3)	1·6 ± 0·6	<0·01
Benzo[a]pyrene (3)	65·5 ± 2·4	NS
Phenobarbitone (3)	63·4 ± 3·6	NS

Numbers of animals are shown in parentheses. Compounds were given as described under methods.

The failure of ethanol to affect the hydroxylation of acetanilide and biphenyl, both of which are aromatic hydroxylations effected through the microsomal mixed function oxidase system (Mitoma, Posner & others, 1956), suggests that amphetamine hydroxylation may differ from the usual microsomal mixed function oxidation. Microsomal oxidations are generally induced by pretreatment with phenobarbitone and some carcinogenic hydrocarbons (Conney & Burns, 1962). However, Groppetti & Costa (1969) failed to induce amphetamine hydroxylation with 3-methylcholanthrene (20 mg/kg) or with phenobarbitone (1·5 mg/rat twice daily for four days) although Lewander (1969) reported a significant increase in hydroxylation after phenobarbitone (80 mg/kg, daily for five days). In the present work neither phenobarbitone (80 mg/kg, daily for three days) nor benzo[a]pyrene (2 mg/kg, daily for three days) produced significant alteration of amphetamine hydroxylation. However, known inhibitors of microsomal hydroxylation (SKF 525-A, DPEA, and Lilly 18947) (Kato, Vassanelli & Chiesara, 1962) did produce marked inhibition of amphetamine hydroxylation *in vivo*.

Dingell & Bass (1969) were able to demonstrate amphetamine metabolism by liver perfusion, but found no metabolism by the microsomal fraction of rat liver. Preliminary observations in this laboratory have confirmed these results.

Amphetamine hydroxylation thus differs from known microsomal hydroxylations by not being induced by phenobarbitone and carcinogenic hydrocarbons. It resembles microsomal hydroxylations by being inhibited by SKF 525-A and other mixed function oxidase inhibitors. It is further characterized by its dramatic and long-lasting inhibition by ethanol. Investigation of this unique aromatic hydroxylation is continuing.

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The biliary metabolism of butylated hydroxytoluene (3,5-di-t-butyl-4-hydroxytoluene) and its derivatives in the rat

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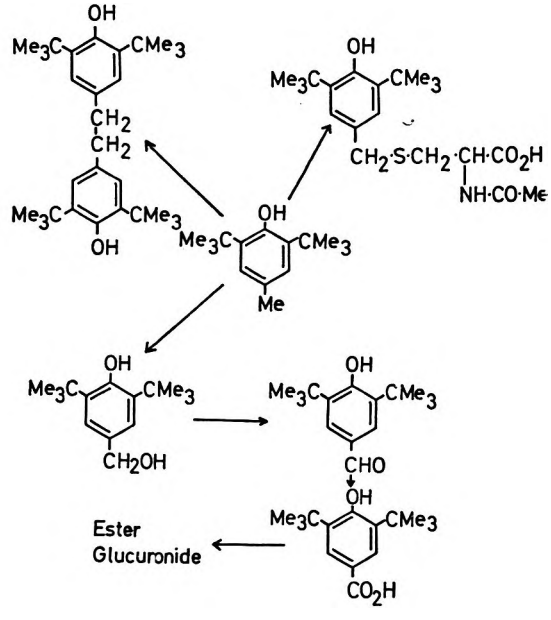
The biliary metabolism of 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT–CH_2OH), 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT–CHO) and 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT–COOH) after parenteral administration has been examined in the rat and compared to that of 3,5-di-t-butyl-4-hydroxytoluene. Quantitative excretion and chemical examination of bile showed that in the enterohepatic circulation BHT–COOH or its ester glucuronide is the recirculating compound from the four compounds studied. Biliary excretion data are also presented for 1,2-bis(3,5-di-*t*-butyl-4-hydroxyphenyl)ethane.

The metabolism of the widely used antioxidant butylated hydroxytoluene (3,5-di-t-butyl-4-hydroxytoluene; BHT) has been reviewed by Hathway (1966). The enterohepatic circulation of its metabolites in the rat was suggested by Daniel & Gage (1965) and demonstrated by Ladomery, Ryan & Wright (1967a), who showed that the metabolites were rapidly excreted into the bile, and relatively slowly into the urine and faeces. The biliary metabolites (Fig. 1) in the rat were later shown to be 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT–CHO), 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT–CH_2OH), 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT–COOH) and 1,2-bis-(3,5-di-t-butyl-4-hydroxyphenyl)ethane (BB) (Ladomery & others, 1967b.)



These findings correlated with urinary metabolites of BHT in the rat and rabbit (Dacre, 1960; Akagi & Aoki, 1962; Wright, Akintonwa & others, 1965). A previously unidentified metabolite in rat urine and bile was shown by Daniel, Gage & Jones (1968) to be *S*-(3,5-di-t-butyl-4-hydroxybenzyl)-*N*-acetylcysteine.

We now report on the biliary metabolism of BHT, BHT-CH_2OH, BHT-CHO, BHT-COOH and B-B in the rat. BHT-COOH was identified as the recirculating compound in the enterohepatic recirculation in the rat. Some additional urinary excretion data are reported.

EXPERIMENTAL

Metabolic experiments

White male Wistar rats (290–350 g), were given doses of [^{14}C]labelled BHT (or derivatives) in aqueous ethanol by intravenous or intraperitoneal injection. Urine and faeces were collected in metabolism cages fitted with all glass separating devices. Bile was collected through a biliary cannula for 6 to 8 h from rats under urethane anaesthesia.

Chromatography

Silica gel G (E. Merck & Co.) was used for both qualitative and preparative thin-layer chromatography (TLC). Various solvent systems used were: A, chloroform–methanol (49 : 1); B, light petroleum b.p. 60–80°; C, light petroleum b.p. 60–80°–ether (2 : 1); D, n-propanol–chloroform (7 : 3); E, methanol–chloroform (2 : 3); F, n-butanol–ethanol–water (2 : 1 : 1). Visualization of BHT and its derivatives was achieved with Gibb's reagent (0·5% 2,6-dichlorobenzoquinone-4-chloroimine in ethanol); glucuronides were detected with naphthoresorcinol reagent (0·3% naphthoresorcinol in ethanol–phosphoric acid (5 : 1). Iodine vapour, and rhodamine (1% in ethanol) viewed under ultraviolet light (254 nm) were used as general reagents.

Methods

Peroxide-free ether was used at all times. All reactions and *work-up* procedures were in a nitrogen atmosphere. Hydrolysis was effected by refluxing with N hydrochloric acid for 0·5 h, or by bacterial β-glucuronidase (Sigma type II) in 0·05M phosphate buffer, pH 7 at 37°.

Reverse isotope dilution analysis involved addition of unlabelled compound to the biological extract before work-up, and purification by preparative TLC. Other radiochemical assays were made on qualitative TLC chromatograms by counting after individual collection of the spots, suspension in scintillation fluid with Cab-O-Sil (Packard Instrument Co.).

"Free-phenols" refer to those compounds extractable from the biological sample by continuous ether partitioning at pH 6.

Materials

The following compounds, used as TLC standards and for reverse isotope dilution, were prepared or obtained from the source indicated: 3,5-di-t-butyl-4-hydroxytoluene (BHT) and bis(3,5-di-t-butyl-4-hydroxybenzyl)ether (Shell Chemical Co., Sydney); 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT-CH_2OH), 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT-CHO), 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT-COOH) were

synthesized according to Ladomery & others (1967b); 1,2-bis(3,5-di-t-butyl-4-hydroxyphenyl)ethane (B-B), 3,3′, 5,5′-tetra-t-butyl-4,4′-dihydroxystilbene (BHT-hydroxystilbene), 3,3′,5,5′-tetra-t-butylstilbene-4,4′-quinone (BHT-stilbenequinone) were prepared according to Cook (1953).

The following [^{14}C] labelled compounds were used: [^{14}C]BHT randomly labelled in the t-butyl groups was obtained from New England Nuclear Corporation, Boston with specific activity $6{\cdot}55 \times 10^6$ d/min mg^{-1} or $2{\cdot}85 \times 10^7$ d/min mg^{-1}.

[^{14}C]BHT-CHO was synthesized by bromine oxidation of [^{14}C]BHT (Coppinger & Campbell, 1953) and purified by preparative TLC (system A). The specific activity after diluent addition was $5{\cdot}58 \times 10^5$ d/min mg^{-1}.

[^{14}C]BHT-CH_2OH was prepared from [^{14}C]BHT-CHO by sodium borohydride reduction and purified by preparative TLC (Solvent A) giving a specific activity after diluent addition of $6{\cdot}19 \times 10^5$ d/min mg^{-1}.

[^{14}C]B-B. Areas corresponding to B-B, BHT-hydroxystilbene and BHT-stilbenequinone were isolated during preparative TLC used to isolate BHT-CHO. Sufficient BHT-stilbenequinone was isolated to reduce with lithium aluminium hydride in tetrahydrofuran (Bohn & Campbell, 1957). Preparative TLC (solvent A) of the product afforded [^{14}C]B-B with a specific activity after diluent addition of $5{\cdot}94 \times 10^5$ d/min mg^{-1}.

The aldehyde, alcohol, and diphenylethane were shown to be radiochemically pure by TLC in solvent systems A and B, A and C, and C respectively.

[^{14}C]BHT-COOH was prepared biosynthetically from 0–8 h bile of rats receiving an intravenous dose of [^{14}C]BHT. Diluent BHT-COOH was added to the bile and isolated by chloroform extraction after refluxing for 0·5 h with an equal volume of 2N hydrochloric acid. The crude acid was purified by preparative TLC using solvents D, E, and C, and the product, specific activity $5{\cdot}47 \times 10^5$ d/min mg^{-1}, was shown to be radiochemically pure (solvent systems A and F).

Radioactivity measurements

Radioactivity measurements were made using a Packard Scintillation Spectrometer, Model 3314, equipped for automatic external standardization. The scintillation fluid consisted of 0·15% PPO and 0·005% POPOP in 10% absolute ethanol in toluene. Aliquots of liquid specimens were counted directly using 10 ml of scintillation fluid; solution of dried finely ground faeces was achieved by digestion of an aliquot with Hyamine hydroxide solution (0·25 ml, Packard Instrument Co. and water 0·1 ml) at 37° overnight. Following addition of fluid, samples were stored in the dark for 24 h before counting.

RESULTS

The hourly excretion of [^{14}C]labelled biliary metabolites of BHT, BHT-CH_2OH, BHT-CHO, BHT-COOH and B-B for 6 h after single intravenous doses is shown in Table 1. Table 2 shows the 2 hrly excretion of the same compounds (excluding B-B) for 8 h after single intraperitoneal doses. Both sets of data show that these compounds are rapidly absorbed, metabolized and excreted in the bile. While the rate of biliary excretion after dosing with the carboxylic acid is greater after intravenous injection, the bulk being excreted in the first hour, the total percentage excreted appears to be independent of the parenteral administration route chosen. By contrast, the total biliary excretion after BHT or BHT-CHO administration is rather less after intra-

Table 1. *Hourly biliary excretion of radioactive metabolites (% dose) during 6 h following single intravenous doses of BHT and related compounds*

Time (h) after dosing	Compound BHT* (100 μg)	BHT–CH_2OH (102 μg)	BHT–CHO (100 μg)	BHT–COOH (112 μg)	B–B (109 μg)
1	46·6 ± 7·5	39·4 ± 8·0	63·1 ± 12·4	78·3 ± 9·8	26·5 ± 7·6
2	25·0 ± 8·0	12·1 ± 3·6	16·1 ± 4·8	1·9 ± 0·1	10·5 ± 3·6
3	12·6 ± 2·6	8·6 ± 5·7	4·2 ± 2·4	0·5 ± 0·1	
4	4·7 ± 1·8	4·2 ± 1·4	2·7 ± 1·9	0·2	
5	2·9 ± 0·6	2·6 ± 0·7	1·0 ± 0·7	0·1	3·3 ± 1·0
6	1·9 ± 0·4	2·1 ± 0·4	0·9 ± 1·2	0·1	
Totals	93·7 ± 11·6	69·2 ± 4·4	88·0 ± 4·5	80·8 ± 9·8	40·3 ± 5·8

* From Ladomery & others (1967a).
Results are the mean values for six rats except for BHT–CHO for which four animals were used. Standard deviations are shown.

peritoneal dosage than after intravenous dosage. The biliary excretion of the alcohol, about 70% of the administered dose, appears to be unaffected by its mode of administration.

The biliary excretion of radiolabelled metabolites of the alcohol, aldehyde and carboxylic acid was also examined for 6 h, 5 days after intraperitoneal administration of these compounds. Table 3 shows the percentage of dose excreted hourly. These results are comparable to those of Ladomery & others (1967a), who found that about 10% of an intraperitoneal dose of BHT was excreted over 6 h, 4 days after dosing.

The total overall excretion pattern of BHT and its related compounds excreted in urine and faeces for 5 days, and bile collected 120–126 h after dosing with a single intraperitoneal dose were examined (Table 4). Although biliary excretion for 120–126 h after intraperitoneal dosage of BHT was not measured, the results of Ladomery & others (1967a) indicate it would not be significantly different from that of its metabolites for this period. There are no significant differences in the total radioactivity excreted over 126 h after dosing, about 70% of the dosed radioactivity for each compound being recovered.

The chemical identity of the metabolites of BHT and related compounds in bile, urine and faeces was examined using reverse isotope dilution analyses, and thin-layer co-chromatography. The Rf values of some of the reference compounds are in Table 5. The predominant metabolite in all biological extracts was the carboxylic acid or its ester glucuronide.

Table 2. *Two hrly biliary excretion of radioactive metabolites (% dose) during 8 h after single i.p. doses of BHT and related compounds*

Time (h) after dosing	Compound BHT* (100 μg)	BHT–CH_2OH (200 μg)	BHT–CHO (300 μg)	BHT–COOH (560 μg)
2	31·5 ± 6·4	57·6 (54·2–61·0)	38·6 (35·4–41·8)	67·1 (62·6–71·5)
4	14·1 ± 1·7	10·1 (9·0–11·1)	12·9 (9·6–16·2)	15·8 (12·9–18·6)
6	6·2 ± 0·9	4·7 (4·3–5·0)	5·6 (4·7–6·4)	3·4 (1·8–5·0)
8	not measured	2·2 (1·9–2·5)	4·2 (3·8–4·6)	1·4 (0·5–2·2)
Totals	51·8 ± 6·6	74·5 (72–77)	61·3 (60–63)	87·6 (86–88)

* From Ladomery (1967a) showing ± standard deviation for six animals.
( ) range of values for two rats.

Table 3. *Hourly biliary excretion of radioactive metabolites (% dose) during 6 h 5 days after single intraperitoneal doses of compounds related to BHT*

Time (h) after dosing	Compound BHT-CH_2OH (102 μg; 8*)	BHT-CHO (100 μg; 5)	BHT-COOH (106 μg; 4)
121	2·3 ± 1·0†	2·0 ± 1·2	2·8 ± 1·5
122	1·5 ± 0·5	1·1 ± 0·7	
123	1·2 ± 0·4	0·7 ± 0·5	2·3 ± 1·2
124	1·0 ± 0·1	0·5 ± 0·3	
125	0·9 ± 0·1	0·2 ± 0·1	2·0 ± 1·5
126	0·9 ± 0·2	0·3 ± 0·1	
Totals	7·9 ± 0·9	4·7 ± 2·2	7·2 ± 4·0

* No. of animals.
† ± Standard deviation.

Little attention was directed to the metabolic products of [^{14}C]BHT except in faeces collected during the 5 day interval after intraperitoneal dosage. Reverse isotope dilution experiments indicated that of the 50% of the dose appearing in the faeces, 40% was free BHT-COOH, and only 0·7% was unchanged BHT. Besides small amounts of nonpolar metabolites a further 40% of the excreted radioactivity was present as conjugated metabolites.

Only the biliary metabolites of [^{14}C]BHT-CH_2OH were examined, the urinary and faecal excretion having been examined by Wright & others (1965). The carboxylic acid, BHT-COOH, was the major metabolite. Of the total radioactivity in 0–6 h bile, 14% was present as free phenols, BHT-COOH predominating, while the ester glucuronide of BHT-COOH was the major component in the remaining metabolites. The only other metabolite detected was a small amount of water-soluble material which was not examined further. Acid hydrolysis, followed by TLC showed that no unchanged BHT-CH_2OH was present. Similar results were obtained after intravenous and intraperitoneal dosage.

In 120–126 h bile from rats receiving intraperitoneal doses of BHT-CH_2OH, traces of the alcohol, aldehyde and BHT-dimers were detected. However the major metabolites were BHT-COOH and its ester glucuronide.

Unchanged BHT-CHO was detected (2% of dose) in the 0–6 h bile of rats given [^{14}C]BHT-CHO (2338 μg) intravenously. Isotope dilution analysis indicated that

Table 4. *Total recovery of radioactive metabolites (% dose) of BHT and related compounds over 126 h after single intraperitoneal doses*

	Compound BHT* (100 μg)	BHT-CH_2OH (102 μg)	BHT-CHO (100 μg)	BHT-COOH (1060 μg)
Urine	32·0 ± 1·2 (10)	14·8 ± 6·9 (4 pairs)	35·2 ± 7·3 (3 pairs)	45·1 ± 7·9 (3 pairs)
Faeces	36·9 ± 1·2 (6)	48·6 ± 4·5 (4 pairs)	28·7 ± 7·6 (3 pairs)	17·4 (2 pairs)
Bile (120–126 h) ..	—	7·9 ± 0·9 (8)	4·7 ± 2·2 (5)	7·2 ± 4·0 (4)
Total.. ..	68·9 ± 1·4	71·3 ± 8·3	68·8 ± 10·8	69·7

± Standard deviation. ( ) No. of animals.
* Ladomery & others (1967a).

Table 5. *Rf values of BHT and its metabolites*

Compound	Rf System A	Rf System B
BHT	0·98	1·0
BHT–CH_2OH ..	0·60	0·50
BHT–CHO	0·85	0·70
BHT–COOH ..	0·15	0·20
BHT-Dimers ..	0·95	0·90–0·95

free BHT-COOH (4% of dose) was present together with conjugated carboxylic acid (57% of dose). These quantities represent 7·8 and 84% of the biliary radioactivity. Acid hydrolysed urine of rats given intraperitoneal [^{14}C]BHT-CHO showed the presence of BHT-COOH (80% of urinary radioactivity) and unchanged aldehyde (1–2%) by TLC analysis. Faeces collected over the 5 days following dosage contained unchanged aldehyde (4% of dose by TLC analysis) while 82% of the radioactivity was present as the free carboxylic acid as demonstrated by reverse isotope dilution analysis.

Bile of rats receiving injections of [^{14}C]BHT-COOH revealed the presence of the free acid and its ester glucuronide as the only metabolites.

Solvent extraction of bile after intravenous injection of [^{14}C]B-B indicated that 3% of the dose (7% of biliary radioactivity) was present as free phenol. Ether extraction at low pH removed an additional 40% of metabolites. TLC investigations of the ether soluble fractions suggested that the metabolites were also dimers. No free BHT-COOH, or its ester glucuronide was detected in these experiments.

DISCUSSION

The 6 h biliary excretion data following injection of low doses of BHT and related compounds indicate that with the exception of the diphenylethane (B-B) these compounds are all rapidly metabolized and excreted in the bile. Examination of the hourly excretion of radioactivity (Table 1) suggests that the rate limiting factor affecting elimination of these compounds or their metabolites into the bile is the rate of oxidation to the carboxylic acid. The total 6 h excretion of the alcohol is significantly ($P < 0·05$) less than that of the parent compound, the aldehyde and the acid. The latter compounds are about 80–90% eliminated in the bile in 6 h, compared to the 70% elimination of BHT-CH_2OH.

The small but significant difference in the total label excreted after intravenous injection of BHT and BHT-CH_2OH may arise through pharmacokinetic factors, or through differences in the oxidation pathways followed by these two compounds.

The biliary excretion pattern after intraperitoneal injection of small doses of BHT and related compounds (Table 2) differs from that after intravenous dosage, suggesting that factors such as precipitation and absorption from the peritoneum, and storage of the drug in the body are important in the metabolism of these compounds after intraperitoneal dosage. However, quantitation of metabolites in late bile, 120 h after intraperitoneal dosing with BHT-CH_2OH, BHT-CHO and BHT-COOH (Table 3) indicated no large differences in the amount of radioactivity excreted in a 6 h period. The proportions found are not substantially different to those found by Ladomery & others (1967a) after intraperitoneal administration of BHT.

In total excretion studies over 5 days with all compounds except B-B, substantial agreement was found with the results for BHT of Tye, Engel & Rapien (1965) (Table 4). For low doses of the compounds tested there were no significant differences in the totals of 5 days urinary and faecal excretion and 120–126 h biliary excretion, about 70% of the administered radioactivity being recovered over 5 days. There were individual differences in the ratio of urinary to faecal excretion over this period, faecal excretion appearing more important for doses of BHT-COOH. While these differences appear significant it is unwise to draw any conclusion from them because the rats used were not monitored for the constancy of their nutritional status and urinary pH.

The lack of differences in the overall excretion pattern and the biliary excretion of BHT and related compounds, strongly suggested that the compound or compounds responsible for the enterohepatic circulation of radioactivity following [^{14}C]BHT administration must be common to the metabolic pathways of all these compounds. Only BHT-COOH or its metabolites could reasonably be suspected since BHT-COOH and its ester glucuronide are known to be the main metabolic endproducts of BHT in bile (Ladomery & others, 1967a) and BHT-CH_2OH in urine (Wright & others, 1965).

In verification of this, the major metabolite found in early bile after parenteral administration of BHT-CH_2OH, BHT-CHO and BHT-COOH was the free acid or ester glucuronide, the latter usually predominating. Examination of late bile after acid hydrolysis again showed the carboxylic acid to be the major radioactive component. Similar results were obtained by Ladomery & others (1967b) for late bile after BHT administration. Variations in the ratio of free acid to ester glucuronide in bile, urine and faeces are probably caused by differing degrees of hydrolysis in the large intestine, or during storage and work-up. As would be expected, less free carboxylic acid than the glucuronide ester is present in the bile. This conclusion verifies those of Daniel & others (1968) about the nature of the recirculating compound in BHT recirculation.

The biliary excretion of the diphenylethane (B-B) contrasts with that of the other compounds studied. Amongst the metabolites, BHT-COOH or other fragments were not recognized, the bulk of metabolites appearing as BHT dimers. This contrast with the metabolism of bis-(4-hydroxy-3,5-di-t-butylphenyl)methane(Ionox 220) and bis(3,5-di-t-butyl-4-hydroxybenzyl)ether(Ionox 201) (Hathway, 1966).

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Microcrystalloptic tests for lysergic acid diethylamide and other hallucinogens

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Microcrystallopic tests for LSD, *NN*-diethyltryptamine, *NN*-dimethyltryptamine, bufotenine, psilocin, psilocybin and STP are described. The tests are recommended in conjunction with other analytical techniques if applied for the forensic identification of hallucingens.

Many analytical techniques for the separation and identification of hallucinogens have been reported. They are mainly based on thin-layer chromatography (TLC), gas-chromatography, spectrofluorometry or infrared spectroscopy (e.g. Genest & Farmilo, 1964; Dal Cortivo & Broich, 1966; Look, 1968; Genest & Hughes, 1969; Andersen, 1969; Katz, Tadjera & Aufricht, 1969; Lerner & Katsiaficas, 1969; Mesley & Evans, 1969). Clarke (1969), in his recent handbook pointed out the usefulness of microcrystal tests for identification purposes. While this book contains descriptions of microcrystal tests for several hallucinogens, none are listed for psilocybin, *NN*-diethyltryptamine and LSD. Also results of microcrystalloptic measurements are not given in any of the existing test procedures for hallucinogens.

EXPERIMENTAL

The methods and equipment were those described earlier (Genest & Hughes, 1968b, c; 1969a, b; Genest, Lowry & Hughes, 1969, Genest, 1970). Instead of microrods, used previously, disposable microcaps (Drummond Sci. Co., Broomall, Pa.) of 1 and 3 μl capacity were used with pedestal slides for support of the cover glasses. For TLC the system chloroform–acetone (1 : 4) on Silica Gel G (Merck) plates and elution techniques as described by Genest & Hughes (1968a, b) were used. Hallucinogens (1 μg/ml) were dissolved in 1% acetic acid, except in the case of LSD for which 1% hydrochloric acid was the solvent of choice. Blank tests consisting of equal volumes of reagent and solvent were prepared from all reagents. Again, the set of reagents proposed by Clarke & Williams (1955, 1957) were the major source in the search for useful tests.

RESULTS

The most characteristic tests for each hallucinogen are given in this section with the results of micro-optical measurements and photographs of typical crystals. For LSD some lesser tests are also listed. All tests were made on pure compounds in concentrations as noted. The time after which the crystals were formed and photographed (in brackets) is also given. LSD tests 2, 3 and 7 were checked in aliquots of TLC-eluates of 20 μg bands.

LSD (*lysergic acid diethylamide, lysergide*)

1. Sodium carbonate (5% in water). Rods, in clusters, some pointed and prism-like; Class 5 (descriptive class-designation according to Farmilo & Genest, 1961);

5 μg; 15 min (2 h); moderate birefringence, 2nd order; parallel extinction; $\pm$ sign of elongation (Fig. 1A).

2. Trinitrobenzoic acid (saturated solution in water). Needles, radiating, in tufts and sheaves, Class 3; 5 μg; 5 min (15 min); moderate birefringence, second order; parallel extinction; $\pm$ sign of elongation (Fig. 1B).

3. Potassium tri-iodide (2 g iodine and 4 g KI in 100 ml water). Rods, small, single, some crossed, Class 5; 1 μg; 15 min (30 min); dim birefringence, 2nd order; parallel extinction; indifferent sign of elongation (Fig. 2A). This test worked equally well in acetic acid solution.

4. Sodium phosphate (5% Na_2HPO_4 in water). Rods, in sheaves or very dense aggregates, Class 5; 5 μg; 5 min; moderate birefringence, 2nd order; parallel extinction; $\pm$ sign of elongation.

5. Potassium cyanide (5% in water). Rods and blades, in clusters, Class 5 and 6; 5 μg; 10 min; moderate birefringence, 2nd order; parallel extinction; $\pm$ sign of elongation.

6. Potassium ferrocyanide (5% in water). Grains, round, showing centered black crosses in polarized light, Class 2; 5 μg; 1 h; parallel extinction; + sign of elongation.

7. Thallium bromide/HBr (2 g TlBr suspended in water), Br_2 added dropwise until dissolved, excess Br_2 removed on water bath). Grains, in burrs, Class 2; 5 μg; 30 min; dim birefringence, 1st order; parallel extinction; + sign of elongation.

8. Styphnic acid (5% in water). Grains, round, small and medium sized, Class 2; 5 μg; 1 h; dim birefringence, 1st order; parallel extinction; + sign of elongation.

Sensitivities of the LSD tests are shown in Table 1. The tests were carried out in 1 μl portions of test solution of decreasing concentration.

NN-Diethyltryptamine

1. Potassium cyanide. Rhombs, single, Class 7a; 1 μg; 2 h (2 h); dim birefringence, 1st order; parallel extinction; positive sign of elongation (Fig. 2B).

2. Styphnic acid. Plates and rods, single, Class 7a & 5; 1 μg; 5 min (15 min); moderate birefringence, 2nd order; parallel extinction; indifferent sign of elongation (Fig. 3A).

Table 1. *Sensitivity of microcrystal test for LSD*

Reagent	Sensitivity (μg)
Sodium carbonate	0·8
Trinitrobenzoic acid	0·6
Potassium tri-iodide	0·2
Sodium phosphate	1·0
Potassium cyanide	2·0
Potassium ferrocyanide	2·0
Thallium bromide/HBr	0·8
Styphnic acid	2·0

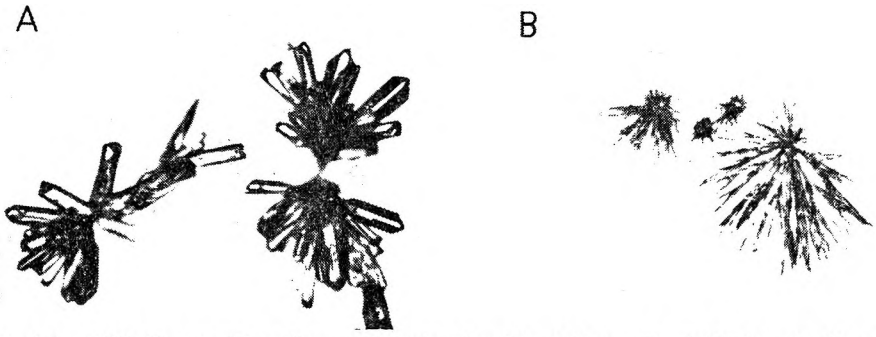

FIG. 1.A. LSD (5 μg) with sodium carbonate after 2 h; 160 ×. B. LSD (5 μg) with trinitrobenzoic acid after 15 min; 160 ×.

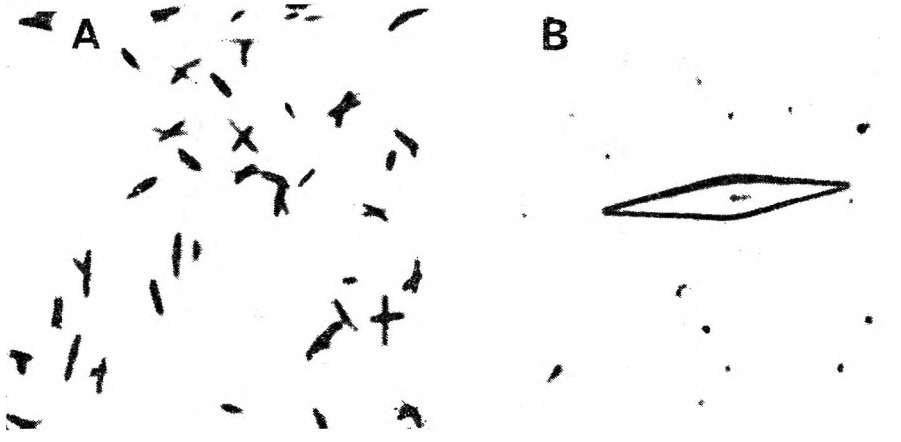

FIG. 2.A. LSD (1 μg) with potassium tri-iodide after 30 min; 400 ×. B. DET (1 μg) with potassium cyanide after 2 h; 160 ×.

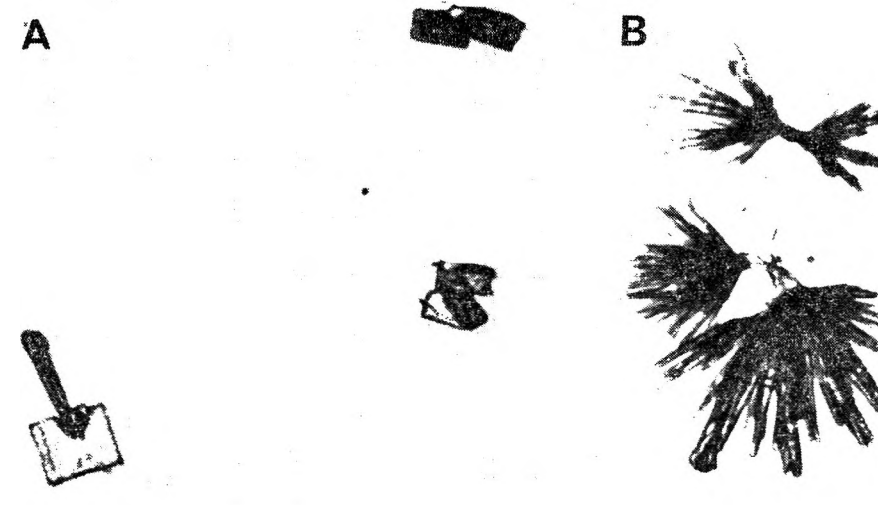

FIG. 3.A. DET (1 μg) with styphnic acid after 15 min; 160 ×. B. DMT (1 μg) with lead iodide after 30 min; 160 ×.

NN-Dimethyltryptamine

1. Lead iodide (30% solution of lead acetate adjusted to pH 6 with acetic acid and saturated with lead iodide). Sheaves of needles, Class 9b; 1 μg; 30 min (30 min); moderate birefringence, 2nd order; parallel extinction; indifferent sign of elongation (Fig. 3B).

2. 5-Nitrobarbituric acid (saturated solution in water). Rods, Class 5; 1 μg; 5 min (15 min); moderate birefringence, 2nd order; parallel extinction; — sign of elongation (Fig. 4A).

Bufotenine

1. Picrolonic acid (saturated solution in water). Tablets; class 7b; 1 μg; 15 min (30 min); moderate birefringence, 1st order; inclined extinction, angle of extinction 28°; indifferent sign of elongation (Fig. 4B).

2. Gold bromide/HCl (5 g $AuCl_3$ and 5 g NaBr in 100 ml conc HCl). Rods and prisms, in irregular aggregates, Class 5; 1 μg; 20 min (30 min); dim birefringence, 2nd order; parallel extinction; indifferent sign of elongation.

Psilocin

1. Picric acid (saturated solution in water). Hairs and needles, Class 4 and 3; 1 μg; 20 min (30 min); moderate birefringence, 2nd order; inclined extinction, angle of extinction 7°; + sign of elongation (Fig. 5A).

Psilocybin

1. Potassium cyanide. Rods and some needles in aggregates, Class 5 and 3; 1 μg; 20 min (30 min); dim birefringence, 1st order; parallel extinction; + sign of elongation (Fig. 5B).

4-*Methyl*-2,5-*dimethoxy*-α-*methylphenethylamine* (*STP*)

1. Picrolonic acid. Needles, branching fine tufts, Class 3; 1 μg; 15 min (30 min); moderate birefringence, 2nd order; parallel extinction; indifferent sign of elongation (Fig. 6A).

2. 2,4,6-Trinito-*m*-cresol (saturated solution in water). Rods in aggregates, Class 5; 1 μg; 10 min (15 min); moderate birefringence, 2nd order, inclined extinction, angle of extinction 150; + sign of elongation (Fig. 6B).

DISCUSSION

The wide-spread misuse of hallucinogens and the concurrent legal implications make unequivocal proof of identity of seized samples containing these compounds a forensic necessity of vital importance. Microcrystal tests play an useful part in identification schemes for confirmatory purposes. While microcrystal tests are mainly used in conjunction with other analytical techniques and after chromatographic purification procedures (Clarke, 1965, 1969; Genest & Hughes 1968b, c; 1969a, b) they apparently can also give valuable results when applied directly to residues obtained by extraction techniques (Moss, 1965). That the evaluation of microcrystal tests based on crystal form is to be done with caution has been noted earlier (Kuhnert-Brandstätter, 1956). Reproducibility is assured only if the experi-

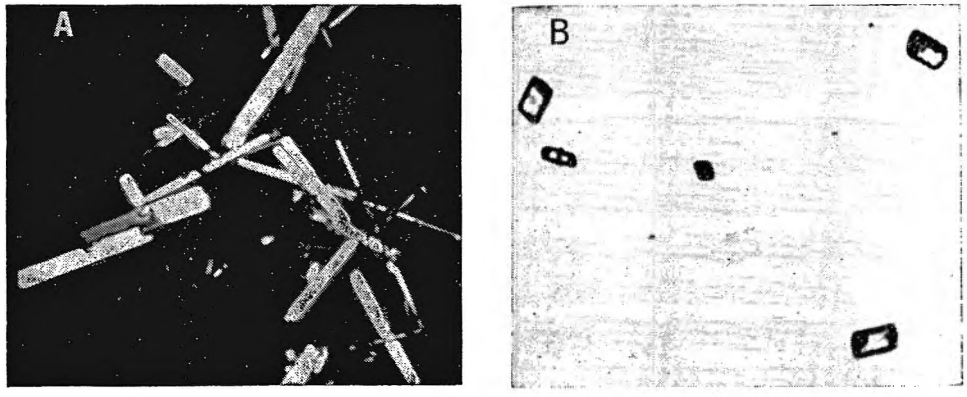

FIG. 4.A. DMT (1 μg) with 5-nitrobarbituric acid after 15 min; 160 ×. B. Bufotenine (1 μg) with picrolonic acid after 30 min; 160 ×.

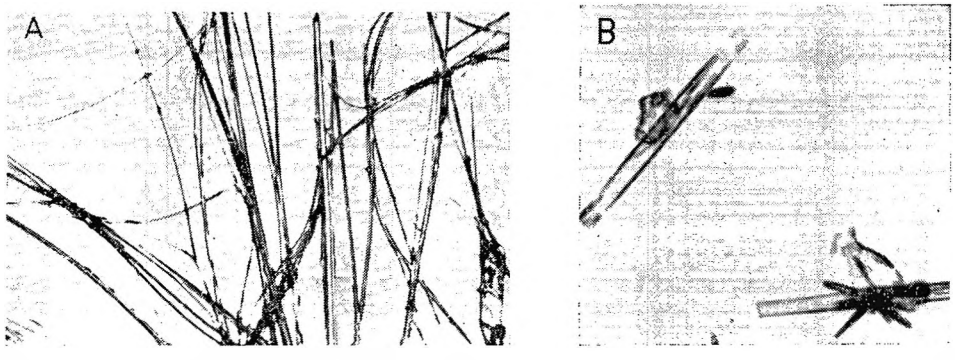

FIG. 5.A. Psilocin (1 μg) with picric acid after 30 min; 110 ×. B. Psilocybin (1 μg) with potassium cyanide after 30 min; 160 ×.

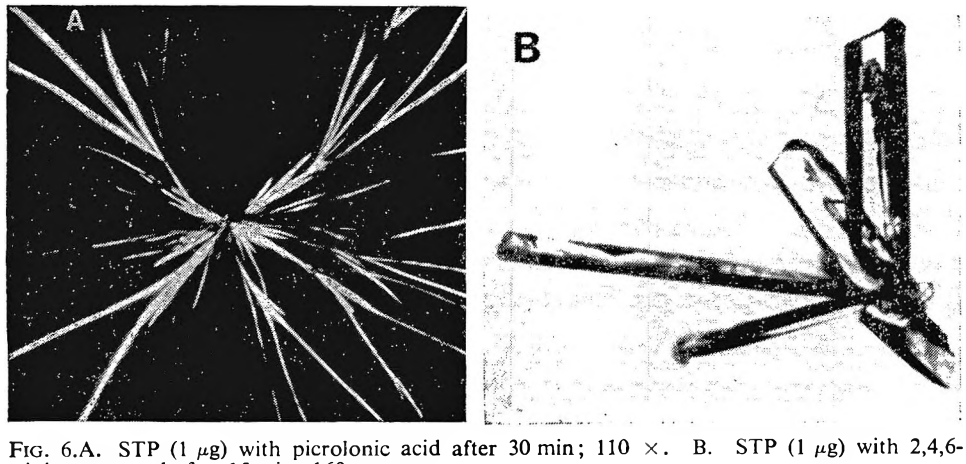

FIG. 6.A. STP (1 μg) with picrolonic acid after 30 min; 110 ×. B. STP (1 μg) with 2,4,6-trinitro-*m*-cresol after 15 min; 160 ×.

mental conditions of solvent, volume and concentration of reagent and reactant, evaporation rate of solvent and time of observation are rigorously controlled. Pedestal slides were preferred in our experiments mainly because of more rapid crystal development. With some reagents, however, especially those consisting of saturated solutions such as the nitrated organic acids, one has to be alert to avoid fallacies due to reagent crystals. For this reason an atlas of blank tests was prepared and consulted regularly to avoid false-positive tests. Thus, when testing LSD in acetic acid with thallium bromide/HBr quite "characteristic" crystals were obtained. These turned out to be probably due to a thallium acetate. The reliability of microcrystal tests can be greatly improved by optical measurements. Where microthermal methods or full crystallographic characterization of crystals cannot be performed, either due to lack of material or experience, measurements of some simple micro-optical properties of the formed crystals in polarized light enhance the value of the tests. In addition to increasing the specificity of microcrystal tests many times (Pozdnyakova & Rozovskii, 1963), these data are sometimes the only means to distinguish closely related alkaloids which give similar crystals with the same reagent (Genest & Hughes, 1968b). In the test for STP with 2,4,6-trinitro-*m*-cresol, crystals of similar form, albeit after a longer time, were obtained in a blank test with the reagent. Micro-optical measurements, however, lead to a clear distinction between blank and test crystals.

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A note on the identification of sulphonamides by thin-layer chromatography

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A method is described for the identification of 25 sulphonamides by thin layer chromatography. Four systems are used, the solvents being (I) chloroform–methanol (4:1), (II) chloroform–carbon tetrachloride–methanol (7:2:1), (III) ethyl acetate–methanol (9:1), (IV) acetone–methanol (4:1), the plates being coated with silica gel G mixed with (I) sodium hydroxide, (II) potassium hydrogen sulphate, (III) water, (IV) sodium hydroxide. The location reagents are (*a*) copper sulphate, (b) *p*-dimethylamino-benzaldehyde, (c) *N*(1-napthyl) ethylenediamine di-HCl, and (d) fluorescein.

Chromatographic methods for the identification of sulphonamides have been given by Klein & Kho (1962), Kho & Klein (1963), Grafe (1964), Fogg & Wood (1965), Lin, Wang & Yang, (1965); Kamp (1966) and Güven & Pekin (1966). Clarke (1968) noted that sulphonamides could be detected on citrate buffered paper chromatograms (Curry & Powell, 1954) but that the Rf values lay too close together to allow satisfactory identification—a common situation when one has a large group of compounds differing but little in chemical structure. The location reagents generally used [*p*-dimethylaminobenzaldehyde or diazotization followed by coupling with alkaline β-naphthol or *N*-(1-naphthyl)ethylenediamine], although extremely sensitive, are not specific, as they give similar colours with other compounds containing a primary arylamino group, nor will they serve to distinguish one sulphonamide from another. To do so requires the use of some reagent which will give different results with different members of the group. An ammoniacal solution of copper has been used by Güven & Pekin (1966) for this purpose.

The reaction between copper sulphate solution and an alkaline solution of a sulphoamide (Sample, 1945) is well known, and has frequently been used to differentiate between these compounds. It was thought that this might serve as a location reaction which would give different coloured spots on the chromatogram with different drugs. Details of such a method are given here.

EXPERIMENTAL

Thin-layer chromatography

Glass plates, 20 × 20 cm, with a 250 μm coat of silica gel G slurried with the appropriate solution, are dryed for 1 h at 110°. Solvent (100 ml) is placed in tanks, 21 × 21 × 10 cm with ends lined with filter paper and allowed to stand for half an hour. The liquid should be changed after each run and the tank re-equilibrated.

Systems

1. Solvent: chloroform–methanol (4:1). Alkaline plates (30 g silica gel G and 60 ml 0·1M sodium hydroxide solution).

2. Solvent: chloroform–carbon tetrachloride–methanol (7 2:1). Acid plates (30 g silica gel G and 60 ml 0·1M potassium hydrogen sulphate solution).

3. Solvent: ethyl acetate-methanol (9:1). Neutral plates (30 g silica gel G and 60 ml water).

4. Solvent: acetone–methanol (4:1). Alkaline plates, as in System 1.

Location reagents

1. Copper sulphate spray. 5% $CuSO_4$ $5H_2O$ w/v, in water.

2. *p*-Dimethylaminobenzaldehyde spray. 1 g of *p*-dimethylaminobenzaldehyde dissolved in 100 ml of ethanol, and 10 ml of concentrated hydrochloric acid added.

3. *N*(1-naphthyl)ethylenediamine dihydrochloride. 0·1% w/v in water.

4. Fluorescein spray. 1·0% w/v in a mixture of acetone and water (3:1).

Procedure

1·0 μl of a 1·0% solution of the sulphonamide in 0·1M sodium hydroxide solution is spotted on the plate and run for 30 min and the plate then dried and sprayed with the copper sulphate solution. (The acid plate in System 2 must then be sprayed with 0·1N sodium hydroxide). The colours of the spots are noted, and the plate oversprayed with the *p*-dimethylaminobenzaldehyde solution, which gives an intense yellow colour with all sulphonamides, except those with substituent groups on N(4), whether they have reacted with the copper sulphate or not. Alternatively, the compounds may be diazotized by exposing the plate to NO_2 fumes and then spraying with *N*(1-naphthyl) ethylenediamine solution to give intense purple spots.

The results are shown in Table 1.

DISCUSSION

The N(4)-substituted compounds succinylsulphathiazole and phthalylsulphathiazole hardly move from the base line in systems 1, 2 and 3. They give brown-purple spots with the copper sulphate spray, but do not react with *p*-dimethylaminobenzaldehyde nor can they be diazotized. They may be identified by their Rf values in system 4. A spot corresponding to the unsubstituted drug, present as an impurity, or formed by decomposition on the plate, is often seen. Phthalysulphacetamide, which does not react with copper sulphate, can be located as an absorbing spot if the plate is finally oversprayed with fluorescein. Sulphasalazine is an orange compound and gives a yellow-orange spot unaffected by the spray reagents used.

Some compounds may run as streaks rather than spots; this is indicated by the letter "s" in Table 1.

Some of the colours with the copper sulphate spray are faint but the alkaline solutions of the drugs, spotted on filter paper and sprayed with copper sulphate, give colours that may be readily recognized.

Table 1. *Rf values of sulphonamides in four solvent systems*

Sulphonamide	Colour	Rf values System 1	System 2	System 3	System 4
Phthalylsulphacetamide	—	0·00	0·00	0·00	0·11
Phthalylsulphathiazole	Brown purple	0·01	0·00	0·00	0·13
Succinylsulphathiazole	Brown purple	0·02	0·00	0·00	0·4
Sulphacetamide	—	0·13	0·25	0·58	0·45s
Sulphadiazine	Brown	0·33s	0·30s	0·55s	0·55s
Sulphadimethoxine	Yellow	0·66	0·45	0·70	0·72
Sulphadimidine	Orange brown	0·67	0·39	0·63	0·69
Sulphaethidole	Green	0·17	0·36	0·50s	0·43s
Sulphafurazole	Greenish brown	0·18	0·34	0·65	0·66
Sulphaguanidine	—	0·26	0·4	0·37	0·62
Sulphamerazine	Brown	0·56	0·34s	0·59	0·61
Sulphamethizole	Green	0·10	0·33	0·43s	0·45
Sulphamethoxazole	Yellow green	0·47	0·41	0·70	0·71
Sulphamethoxydiazine	Purple brown	0·63	0·39s	0·62	0·68
Sulphamethoxypyridazine	Brown	0·68	0·39	0·63	0·71
Sulphamoprine	Orange	0·71	0·49	0·73	0·77
Sulphanilamide	—	0·41	0·14	0·66	0·75
Sulphaphenazole	Light brown	0·57	0·43	0·69	0·76
Sulphapyridine	Light brown	0·58	0·22	0·61	0·69
Sulphaquinoxaline	Yellow green	0·41	0·41s	0·65	0·71
Sulphasalazine	(Orange)	0·7	0·2	0·10s	0·32s
Sulphasomidine	Yellow green	0·40	0·17	0·41	0·42s
Sulphasomizole	Grey brown	0·22	0·31	0·65	0·69
Sulphathiazole	Brown purple	0·44	0·20s	0·50	0·62
Sulphormethoxine	Faint yellow	0·69	0·50	0·69	0·73

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Application of the ΔA method to the determination of morphine

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Tablets, suppositories and ear-drops have been assayed using the ΔA method to eliminate interferences. The absorbance at 298 nm is measured for solutions in acid and alkaline media, the difference (Δ A) being taken as a measure of morphine content. The same method has been applied to suppositories after extraction of the drug from the base. Ear-drops containing phenol in addition to morphine were assayed by measuring ΔA at 288 and 298 nm and evaluating the morphine concentration from a pair of simultaneous equations.

If a compound contains a pH-sensitive auxochrome, its spectrum usually shifts when the pH changes over a suitable interval. In the ΔA method, such a "chemical shift" may be used to cancel the effect of irrelevant absorption (Aulin-Erdtman, 1955).

Morphine exhibits a maximum absorption at 285 nm in 0·1N sulphuric acid and at 298 nm in 0·1N sodium hydroxide (B.P. 1968). In these circumstances, pH-sensitive irrelevant absorption, z, may be cancelled by means of

$$\Delta A_i = (A_b + z)_i - (A_a + z)_i \quad .. \quad .. \quad .. \quad (1)$$

where A_a and A_b are the absorbances of 1 cm layers of acid and alkaline solutions containing the same concentration of morphine in each and the subscript "i" refers to the wavelength, 298 nm at which ΔA is maximum. The concentration of morphine, c_M, is given by:

$$c_M = \Delta A_i/\Delta\alpha_i \text{ where } \Delta\alpha_i = \alpha_{bi} - \alpha_{ai} \quad .. \quad .. \quad (2)$$

and α denotes the absorptivity of morphine.

By combining Vierordt's method and the ΔA method, a mixture of two-known pH-sensitive absorbing substances, A and B, may be determined in the presence of a pH-insensitive irrelevant absorption. Thus, the concentration of A and B can be evaluated from a pair of simultaneous equations of the following form:

$$\Delta A_1 = c_A\Delta\alpha_1 + c_B\Delta\beta_1 \quad .. \quad .. \quad .. \quad (3)$$

$$\Delta A_2 = c_A\Delta\alpha_2 + c_B\Delta\beta_2 \quad .. \quad .. \quad .. \quad (4)$$

The subscripts 1 and 2 refer to wavelengths; ΔA denotes the absorbance difference of the mixture in alkaline and acid media; c_A and c_B are the concentrations of A and B, whilst α and β are their respective absorptivities.

A good choice of wavelengths for a two-substance ΔA method is rather less obvious than for the one-substance ΔA method. Evidently, the error in an assay result based upon the former method which involves four absorbances measured at two wavelengths for solutions of A, B, and their mixture would be greater than that in a result based upon two absorbances measured at a single wavelength in a one-substance ΔA method. Thus, unless the principles of choosing wavelengths are well understood,

that is (i) avoidance of absorption curve slopes whenever possible; (ii) preference for λ_1 and $\lambda_2 = \lambda_{max}$ of the ΔA curves of substances A and B, the method may give unsatisfactory results.

EXPERIMENTAL

Instrument. A Unicam SP 500 photoelectric spectrophotometer.

Assay for tablets. Tablets were prepared, containing 10 mg of morphine sulphate per 0·30 g tablet (B.P. 1968). An accurately weighed quantity of the powder, representing 10–20 tablets, was quantitatively extracted with 0·1N sulphuric acid, filtered into a 100 ml volumetric flask and subsequently made to volume. Two 10 ml portions of the filtrate were diluted to 100 ml using 0·1N sulphuric acid and 0·1N sodium hydroxide respectively. The absorbances of 1 cm pathlengths of both solutions were measured at 298 nm.

Assay for suppositories. Suppositories were prepared, containing 15 mg of morphine hydrochloride per 1·5 g theobroma oil in each suppository. An accurately weighed quantity (10–20 g) of the melted suppositories was dissolved in 60 ml of light petroleum saturated with water. The solution was extracted with 3 × 20 ml of 0·1N sulphuric acid and subsequently washed with 3 × 5 ml portions of acidulated water. The combined aqueous extracts and washings (75 ml) were warmed on a water bath, cooled, and transferred to a 100 ml volumetric flask and made to volume. Final dilutions were prepared and absorbance measurements made by the method described for tablets.

Assay for ear-drops. This preparation contained morphine hydrochloride, 0·75 g and phenol, 1·5 g, made to 20 g (about 15 ml) with glycerol. An accurately weighed quantity (about 1·0 g) of the ear-drops was dissolved in water and final solutions prepared by the method described for tablets. Absorbances were measured at 288 and 298 nm.

To minimize the relative error in ΔA, the concentrations of the final solutions were controlled so that $(A_a + z)_1 + (A_b + z)_1$ was less than 1·0 (Junejo & Glenn, 1956; Glenn, 1965).

RESULTS AND DISCUSSION

When compared with the graph of log A versus λ for morphine sulphate, similar graphs for tablets and suppositories were distorted due to the presence of absorbing impurities. On the other hand, the graphs of log $|\Delta A|$ versus $\lambda(\Delta A \neq 0)$ for tablets and suppositories were completely superimposable with that for morphine sulphate, thus showing the irrelevant absorption to be unchanged in the two solvents used.

Table 1. *The determination of morphine in tablets and suppositories*

	Tablets		Suppositories	
Sample	mg added	% recovery	mg added	% recovery
1	8·0	100·25	40·0	96·7
2	10·0	101·20	25·0	98·4
3	16·0	100·25	20·0	99·5
4	20·0	100·00	15·0	98·7
5			10·0	102·0

It was not, therefore surprising that the above ΔA procedures gave mean percentage recoveries of 100·2 ± 0·8% for the morphine sulphate in four separate weighings of the powdered tablets and 99·1 ± 2·4% ($P = 0·05$) for the morphine hydrochloride in five separate weighings of the melted suppositories (Table 1).

By applying Vierordt's method (i) using absorbances and (ii) using absorbance differences, morphine in different concentrations, was determined in ear-drops containing phenol (Table 2). The mean percentage recovery was found to be 105·0 ±

Table 2. *The determination of morphine hydrochloride in ear-drops using Vierordt's method* ($\lambda_1 = 288$ nm and $\lambda_2 = 298$ nm)

Sample	Method mg added	Absorbance % recovery	Absorbance differences % recovery
1*	2·0	106·0	102·5
2*	2·4	107·5	102·9
3*	2·8	107·1	103·6
4	4·0	105·0	101·7
5	6·4	103·6	102·0
6	8·0	103·6	100·1

All samples contained 4·0 mg phenol.

* Low contribution of morphine hydrochloride.

2·7% using the former method (Glenn, 1960) and 102·1 ± 1·3% using the latter method (P 0·05). The poor results from the method using absorbances were due to irrelevant absorption in the glycerol which was cancelled by the method using absorbance differences.

Errors in both methods can also be attributed to (i) overall shifts in the wavelength scale which affect absorbance measurements made on steep slopes in the phenol and morphine hydrochloride absorption curves (Ismail & Glenn, 1964) and (ii) the low contribution of morphine to the mixture's total absorption curve especially, in samples marked with an asterisk (Glenn, 1960).

In the light of a satisfactory literature survey, the authors believe that the extension of the ΔA method to mixtures of two substances is original. The results obtained for the determination of morphine in presence of phenol are encouraging and suggest that the method warrants a careful study over a wide field of applications.

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LETTERS TO THE EDITOR

Photometric evaluation of dye diffusion in the skin

If a local cutaneous lesion is produced by physical or chemical agents, colloidal dyes injected intravenously will diffuse in the cutaneous tissue. This phenomenon, reported by Spagnol (1929), has been used to study capillary permeability changes, after local action of inflammatory agents, or in quantitative evaluation of the protective or antagonistic action of several drugs.

Lockett & Jarman (1958) compared the intensity of the blue dye at an injection site with a series of comparator cards; Miles & Wilhelm (1955) and Jori, Bentivoglio & Garattini (1961) measured the lesion diameters; Parrat & West (1958) used simple visual grading of the response. Recently Judah & Willoughby (1962) described a quantitative method to measure trypan blue after extraction from a given cutaneous area. Young (1964), Ankier & Whiteside (1969) used a spectrophotometric method based on extraction in alkaline medium for the determination of protein-bound dye from tissues. Nevertheless, spectrophotometric methods, though sensitive, are laborious in pharmacological screening; maceration of tissue samples and centrifugation, essential for clear solutions, represent the principal difficulties.

We describe here a method for the direct measurement of the dye in rat skin with the technique used in the reading of electrophoretic strips.

Local lesions were produced by injecting inflammatory agents (like 5-hydroxytryptamine, bradykinin or histamine) by the intradermal route, in different doses for each rat and constant volume of 0·1 ml per dose, immediately after the dye. The injection sites were placed 30 mm from each other, in two rows parallel and 15 mm from the spinal cord. In different animals the injection sites of a drug were differently disposed to avoid injecting the same drug in the same area. The skin of each rat, dried and cleaned of all the subcutaneous tissue, was cut into longitudinal strips, each containing 2 or 3 blue spots. These strips were embedded for 3 h in clarifying solution [tricresylphosphate–light petroleum (40–60°)–acetone; 70:10:20] and then mounted between two glass slides. Readings were taken on a photometer for electrophoretic strips with 640 nm filter. The values obtained, expressed in mm^2, represent the integration of the absorbance and the area of a single blue spot.

Statistical analyses made comparatively on the results of our technique and that of Judah & Willoughby demonstrated the high sensitivity of the proposed method and its suitability for the evaluation of local capillary permeability changes.

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Non-antigenicity of a salicylamide derivative of aspirin

Weiner, Rosenblatt & Howes (1963) have shown circulating antibodies against aspirin in sera of patients allergic to aspirin and rabbits immunized with aspirin-protein conjugates, using the tanned red cell agglutination technique. One remarkable feature of aspirin hypersensitivity is that individuals who react to aspirin rarely do so to salicylates. It has been shown (Schwartz & Amidon, 1966) that aspirin can react with amines to produce a salicylamide derivative whereas salicylic acid does not, though a very small proportion of the original aspirin reacts with amines to produce the salicylamide derivative. Schwartz & Amidon (1966) considered that if salicylamide derivatives could be proved to be the antigenic determinant in hypersensitivity to aspirin, it might offer a possible explanation for the lack of hypersensitivity to salicylates by patients known to be allergic to aspirin. We now report that the salicylamide derivative of aspirin does not act as an antigen.

N-Salicyloylglycine was prepared by refluxing glycine (0·03 mol) with sodium hydroxide (0·08 mol) and phenyl salicylate (0·02 mol) for 4 h, the mixture was cooled, acidified and precipitates crystallized from chloroform–methyl acetate (3:1).

The test solution of *N*-salicyloylglycine was prepared in sterile isotonic saline solution in a concentration of 1% amino-acid. Two groups of rabbits, each animal being more than 2·5 kg were treated. One was immunized with the test solution and the other with the test solution blended with an equal volume of complete Freunds' adjuvant (Feinberg & Malkiel, 1951). The animals were bled periodically for the detection of antibodies.

The indirect haemagglutination test using tanned sheep red blood cells (Stavitsky, 1954) and gel precipitation techniques were used for detection of circulating antibodies against *N*-salicyloylglycine in the sera. A group of guinea-pigs was passively sensitized by intraperitoneal injection of 5 ml of immunized rabbit serum and challenged after 48 h by intravenous injection of the derivative. Another group of guinea-pigs was actively immunized and challenged after 2 weeks (Feinberg & Malkiel, 1951).

No circulating antibodies were demonstrable in the sera of immunized rabbits. Guinea-pigs immunized either passively or actively did not show sign of anaphylaxis when challenged.

Hence, it seems that the salicylamide derivative is not antigenic by itself. Different authors (Weiner, Rosenblatt & Howes, 1963; Wicher, Schwartz & others, 1968) have shown that when rabbits were immunized with aspiryl conjugates of high molecular weight proteins like crystalline egg albumin or bovine γ-globulin, circulating antibodies could be demonstrated in the immune sera to very high titres. It seems most probable that a complex protein molecule may be responsible for imparting antigenicity to aspirin-protein conjugates rather than a simple amine which reacts with aspirin to give salicylamide derivatives.

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Salicylates and gastric juice

The suggestion that gastrointestinal bleeding resulting from oral administration of aspirin may be due in part to precipitation of a protective glycoprotein component of gastric juice by the aspirin has been made by Rainsford, Watkins & Smith (1968). This was supported by the observation that mucin extracts of pig stomachs were partially precipitated by 50 mM sodium salicylate at pH 3·6.

We have undertaken a series of experiments to establish whether salicylates cause any precipitation of glycoproteins in human gastric juice and saliva.

The conditions under which Rainsford & others (1968) observed the precipitation of pig mucins were used: pH 3·6 and a final salicylate concentration of 100 mM, which approximates to the concentration of salicylate in a human stomach after ingestion of 20 grains of sodium salicylate. Sodium salicylate, which at pH 3·6 is soluble up to concentrations of 0·5M, was used rather than acetylsalicylic acid which is insoluble.

The secretion was dialysed against 0·01M sodium acetate buffer, pH 3·6, in 0·15M NaCl. Samples (300 μl) of the dialysed secretion were mixed with 75 μl of the same buffer or with 75 μl of 0·5M sodium salicylate in the same buffer. The increase in absorbance at 420 nm after 10 min at 25° was taken as a measure of the precipitation. All measurements were done in duplicate.

Samples of gastric juice, both in the resting state and for 1 h after the injection of 50 mg of histalog were obtained from a normal volunteer (Group O, non secretor). During the aspiration of gastric juice all saliva produced was expectorated and collected. The gastric juice was neutralized with N NaOH immediately and both gastric juice and saliva were spun at 1800 *g* for 15 min. The supernatants were concentrated by ultrafiltration, dialysed against 0·1M sodium acetate buffer, pH 3·6, in 0·15M NaCl and again centrifuged at 1800 *g* for 15 min; a small amount of insoluble material separated in each case.

The interaction of the supernatants with sodium salicylate is shown in Fig. 1A. Both the resting gastric juice and saliva gave a precipitate with sodium salicylate

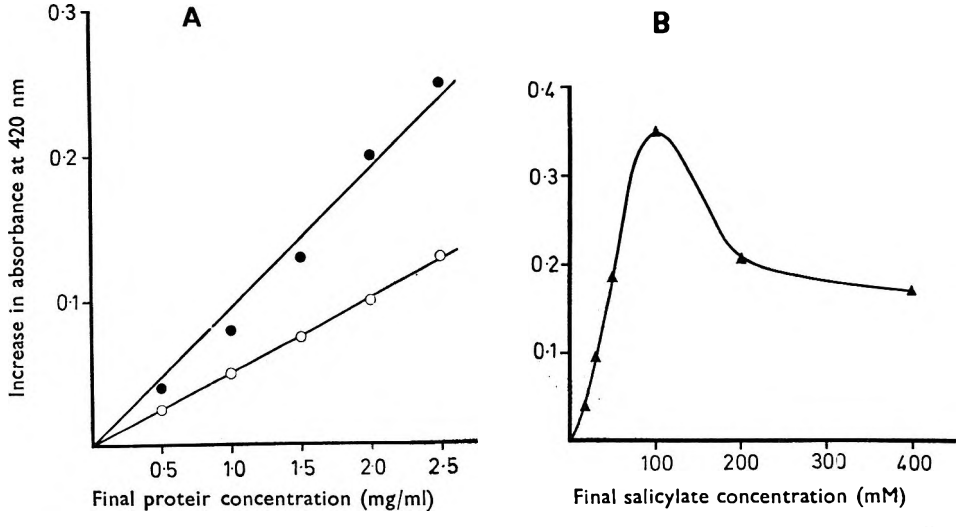

FIG. 1.A. Interaction of 50 mM sodium salicylate with gastric juice and saliva at pH 3·5. B. Effect of increasing concentrations of sodium salicylate on the precipitation of saliva at a concentration of 3 mg/ml protein.

over the concentration range tested, 0–2·5 mg/ml protein. Protein was measured by the method of Lowry, Roseborough & others (1951) as bovine serum albumin.

Increasing concentrations of sodium salicylate caused a linear increase in the precipitation of saliva at a protein concentration of 3 mg/ml. At higher salicylate concentrations there was less precipitation (Fig. 1B).

No interaction of post-histalog gastric juice over the range 1–4 mg/ml protein was detected with the final concentrations of 50, 100, 200 and 300 mM sodium salicylate.

It is possible that the precipitation of resting gastric juice, but not post-histalog gastric juice, with sodium salicylate was due to the presence in resting gastric juice of swallowed saliva which was found to interact strongly with sodium salicylate.

Once an interaction between sodium salicylate and both saliva and resting gastric juice had been demonstrated, it seemed important to determine which components of the secretions were involved in the reaction. Accordingly the same experiments were made on gastric juice and saliva samples from volunteers, X and Y, both Group O non-secretors, which were fractionated in a caesium chloride density gradient as described by Creeth & Denborough (1970).

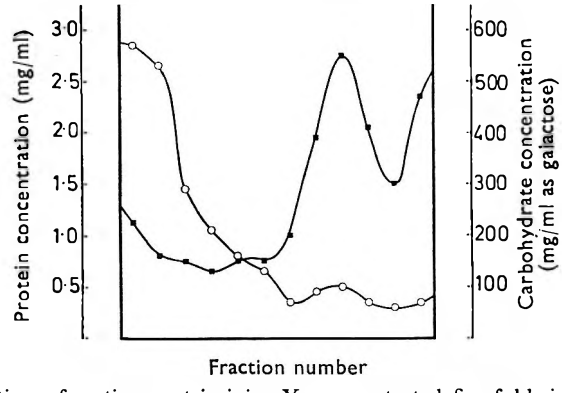

FIG. 2. Fractionation of resting gastric juice X, concentrated five-fold, in a caesium chloride density gradient. Protein ○——○. Carbohydrate ■——■.

In each case a good separation of the glycoprotein from the protein component was achieved. The fractionation of resting gastric juice X is shown in Fig. 2. Fractionation of other secretions gave similar patterns.

The fractions at the glycoprotein peak of each secretion were combined and

Table 1. *Interaction between sodium salicylate* (100 mM) *and fractions from saliva and gastric juice of volunteers X and Y.*

	Protein fraction		Glycoprotein fraction	
	Interaction with salicylate (Δ absorbance) 420 nm	Final concn (mg/ml protein)	Interaction with salicylate (Δ absorbance) 420 nm	Final concn (mg/ml protein)
X. Resting gastric juice	0·100	1·3	0·040	1·3
Y. Resting gastric juice	0·059	2·5	0·010	1·1
X. Gastric juice posthistalog ..	0·010	1·4	0·000	1·3
Y. Gastric juice posthistalog ..	0·000	1·3	0·000	1·4
X. Saliva	0·082	1·4	0·010	0·8
Y. Saliva	1·20	2·1	0·029	0·7

concentrated to give samples having >1·0 mg/ml protein. The fractions at the protein peak of each secretion were also combined and concentrated to give samples having similar concentrations of protein.

The concentrated combined fractions were separately dialysed against 0·01M sodium acetate buffer, pH 3·6 in 0·15M NaCl and the interaction with sodium salicylate at a final concentration of 100 mM was measured turbidimetrically as described. The results are shown in Table 1.

Neither the glycoprotein nor the protein fractions from either sample of post-histalog gastric juice interacted with sodium salicylate, confirming the observations with unfractionated post-histalog gastric juice. However, protein fractions of both resting gastric juice and saliva interacted strongly with sodium salicylate. There was also a very slight interaction with the glycoprotein fraction in both resting gastric juice and saliva.

Although little precipitation of the separated glycoproteins of resting gastric juice or saliva was detected, it is possible that in the whole secretions there may be some coprecipitation of the glycoprotein during precipitation of the protein.

However, to obtain any detectable precipitation of the protein from either gastric juice or saliva the secretion had to be concentrated at least 10 times, and even in the concentrated secretions, the maximum precipitation obtained represented less than 20% of the total protein and glycoprotein. Thus it seems unlikely that in man there would be any significant precipitation of glycoprotein of gastric juice by 100 mM sodium salicylate *in vivo*.

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Effects of desipramine, phentolamine and phenoxybenzamine on the release of noradrenaline from isolated tissues

Adrenergic nerves in isolated tissue incubated with [^{3}H]noradrenaline (^{3}H-NA) take up the amine by an active mechanism, the membrane pump, and incorporate it into the amine storage granules (Carlsson 1966; Hamberger 1967; Jonsson, Hamberger & others, 1969). Field stimulation of isolated tissue is known to cause release of ^{3}H-NA from the adrenergic nerves (Baldessarini & Kopin 1967; Farnebo & Hamberger 1970). The effects of membrane pump blocking and α-receptor blocking drugs on transmitter release and overflow have been examined in several experimental models with divergent results (see e.g. Brown & Gillespie, 1957; Blakeley, Brown & Ferry, 1963; Thoenen, Huerlimann & Haefely, 1964a, b; Boullin, Costa & Brodie, 1967). Field stimulation of isolated tissue was considered an appropriate model for such studies as the stimulation did not affect the circulation in the tissue. We now report the influence of drugs on the ^{3}H-NA release from central and peripheral tissues.

Isolated irides and cerebral cortex slices of standardized size (diameter 3 mm, thickness 0·5 mm) from untreated female rats (Sprague–Dawley, 180–200 g) were carefully prepared. The tissue was incubated at 37° in a modified Krebs-Ringer bicarbonate

medium containing 10^{-7}M ^{3}H-(±)-NA (10 Ci/mmol, New England Nuclear) (see Jonsson & others 1969). After 30 min incubation it was transferred to small stimulation chambers and superfused with ^{3}H-NA-free buffer containing the drug to be tested. After superfusion for 30 min the tissue was stimulated by an electric field (biphasic pulses, 12 mA, 2 ms, 10/s) (Farnebo & Hamberger 1970) for 10 (iris) or 2 (cortex) min and superfused for another 15 min. The superfusate (0·5 ml/min) was collected in 5 min fractions and analysed for total radioactivity by liquid scintillation counting. After the superfusion the tissue was dissolved in Soluene and analysed for total radioactivity. Quenching was measured by re-counting representative samples after the addition of a standard amount of [^{3}H]toluene. The stimulus-induced release was measured as total tritium overflow during the stimulation minus the calculated spontaneous overflow during the same period, and was expressed as per cent of the tritium content in the tissue at the onset of stimulation (calculated by adding the tritium efflux and the tritium content of the tissue at the end of the superfusion). The following drugs were tested: desipramine 10^{-8} — 10^{-6}M; phentolamine 10^{-6} — 10^{-5}M; phenoxybenzamine 10^{-6} — 10^{-5}M.

Field stimulation caused release of radioactivity both from isolated irides and from cerebral cortex slices (see Table 1). This release has been shown to consist mainly of unchanged ^{3}H-NA (Baldessarini & Kopin 1967; Häggendal, Johansson & others, 1970). The fraction of the exogenous ^{3}H-NA overflowing per impulse was calculated to be $2{\cdot}0 \times 10^{-5}$ for iris and $12{\cdot}7 \times 10^{-5}$ for cerebral cortex. Desipramine which is a potent inhibitor of the membrane pump in noradrenaline nerves (Hamberger 1967), caused a rather small increase of the overflow in both tissues. The α-receptor blocking drugs phentolamine and especially phenoxybenzamine 10^{-6}M caused a larger increase of the overflow than desipramine. In irides, phenoxybenzamine 10^{-5}M further increased the overflow to about four times the control. We have found phenoxybenzamine 10^{-5}M, but not 10^{-6}M, to effectively block the membrane pump in isolated irides, and this may explain the present difference between 10^{-5} and 10^{-6}M phenoxybenzamine. In agreement with this assumption the overflow with phenoxybenzamine 10^{-6}M could be augmented by desipramine (Table 1). Also, the increase

Table 1. *The effect of drugs on field stimulation induced release of* [^{3}H]*noradrenaline.* Isolated irides or cerebral cortex slices from untreated rats were incubated with ^{3}H-NA 10^{-7}M, and then superfused for 30 min with ^{3}H-NA-free buffer to which the drugs to be tested had been added. Subsequently the tissue was stimulated for 10 (iris) or 2 (cortex) min and after rinsing for 15 min the radioactivity in the tissue was determined. The stimulation induced overflow of tritium is expressed as per cent of the tritium content in the tissue at the onset of the stimulation (see text) and the values are given as mean ± s.e. Number of observations within brackets.

Drug	Concentration M	Iris	Cerebral cortex
None		11·8 ± 0·5 (37)	15·1 ± 0·9 (28)
Desipramine	10^{-8}	12·3 ± 1·3 (7)	18·2 ± 1·3 (8)
Desipramine	10^{-7}	16·5 ± 1·2 (18)	17·2 ± 1·1 (7)
Desipramine	10^{-6}	18·9 ± 1·0 (11)	20·5 ± 1·9 (7)
Phenoxybenzamine	10^{-6}	32·6 ± 3·2 (15)	26·8 ± 1·7 (8)
Phenoxybenzamine	10^{-5}	49·9 ± 2·8 (12)	25·5 ± 2·3 (6)
Phentolamine	10^{-6}	20·5 ± 0·5 (4)	26·7 ± 2·0 (7)
Phentolamine	10^{-5}	20·2 ± 1·7 (8)	22·5 ± 1·8 (8)
Desipramine + phenoxybenzamine	10^{-7} 10^{-6}	42·1 ± 2·5 (12)	
Desipramine + phentolamine	10^{-7} 10^{-6}	29·8 ± 2·1 (6)	

induced by phentolamine could be potentiated by desipramine. The increased overflows caused by desipramine in combination with α-receptor blocking drugs were in fact larger than the increase caused by desipramine alone.

It has been claimed that phenoxybenzamine increases overflow of noradrenaline by blocking its binding to the α-receptors (Brown & Gillespie, 1957, Boullin & others, 1967). But we have found phenoxybenzamine to cause a markedly increased noradrenaline overflow also in the mouse isolated atrium and a direct effect of this α-blocking drug on the adrenergic nerves, not related to membrane pump inhibition, can thus not be excluded. The present findings might also be explained by an interaction between the adrenergic nerves and the α-receptors which could lead to a variable release (Häggendal 1970). Thus, membrane pump blockade could be expected to cause a marked increase of the overflow as the transmitter is not readily taken up again. However, as the overflow is only slightly increased, it may be assumed that the release from the adrenergic nerves is decreased after inhibition of the membrane pump. When the α-receptors are blocked there is a marked increase of the overflow suggesting that the receptor blockade led to an increased release of the transmitter from the adrenergic nerves, compared to the control. Combination of membrane pump and α-receptor blockade caused the most marked increase of the transmitter overflow but the effect on the release is unclear. Thus, in agreement with Häggendal (1970) the receptor organ seems to be able to influence the amount of transmitter released.

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A possible role for 5-hydroxytryptamine in drug-induced seizures

The pattern of convulsive seizures seen in rats injected with leptazol (pentylenetetrazol) was used to test the anticonvulsive properties of several drugs that affect brain monoamine metabolism. Seizures were measured after 40 mg/kg of leptazol followed by other drug treatment according to the following index. Phase I: no seizure (A) or mild myoclonic head jerk (B). Phase II: clonic-tonic convulsions (C) and loss of righting reflex for 3 s or less (D). Phase III: loss of righting reflex for 5–10 s (E) and prolonged loss of righting reflex, over 2 min (F). Phase IV: death (G). All injections were given intraperitoneally. The latency period before seizure was 75 s (±10 s). When phenobarbitone (6 mg/kg) was given 1 h before leptazol, an increase in the rat seizure threshold was seen (Table 1). Treatment with the peripheral decarboxylase inhibitor Ro 4–4602 [*N*-(DL-seryl-*N*^{1}-2,3,4-trihydroxybenzyl)hydrazine] followed 30 min later by 100 mg/kg of 5-hydroxytryptophan (5-HTP) 1 h before leptazol, resulted in no seizures in 35% of the rats while the remaining 65% had only a mild myoclonic head jerk (Table 1). At the same doses, single injections of either Ro 4–4602 or 5-HTP did not alter the susceptibility of the animal to leptazol compared with the controls.

Table 1. *Relative seizure susceptibility to leptazol after various drugs.* Phase I, indicates no or very mild seizure; Phase II, moderate seizure; Phase III, severe convulsions, Phase IV, death. Numbers indicate % rats; total number of animals are shown in parenthesis. Comparatively good protection in phenobarbitone-treated rats (75% under Phase II) while excellent protection is seen after Ro 4–4602 + 5-HTP (100% Phase I).

	Dose mg/kg	Phase I		Phase II		Phase III		Phase IV
		A	B	C	D	E	F	G
Disulfiram*	3×100	—	—	10 (1)	10 (1)	80 (8)	—	—
α-Methyltyrosine*	3×100	—	—	10 (1)	20 (2)	70 (7)	—	—
Ro 4-4602*	50	—	5 (1)	5 (1)	5 (1)	70 (14)	—	15 (3)
5-HTP*	100		5 (1)	30 (6)	5 (1)	60 (12)	—	—
Phenobarbitone†	6	10 (2)	5 (1)	55 (11)	30 (6)	—	—	—
Ro 4–4602 + 5-HTP‡	50+100	35 (7)	65 (13)	—	—	—	—	—
Leptazol	40	—	—	10 (8)	5 (4)	80 (64)	2·5 (2)	2·5 (2)

* $P = > 0.05$ (n.s.). † $P = < 0.01$. ‡ $P = < 0.001$.

The dopamine-β-hydroxylase inhibitor disulfiram and the tyrosine hydroxylase inhibitor α-methyl tyrosine when given at 100 mg/kg daily for 3 days did not significantly change the rat seizure threshold when compared to leptazol controls. Both these compounds can reduce brain catecholamines by inhibiting the formation of either noradrenaline or dopamine (Musacchio, Kopin & Snyder, 1964; Corrodi & Hanson, 1966).

Similarly, the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (*p*-CPA) was administered for 3 days at 100 mg/kg daily but with leptazol reduced to 35 mg/kg. At this last dose, 45% of the leptazol controls had no seizure, 25% had clonic-tonic convulsions and 30% had brief loss of righting reflex 5–10 s (Table 2). After *p*-CPA, however, the seizure pattern was exacerbated. In addition, 24 h after a strong single dose of *p*-CPA (316 mg/kg) all rats had severe loss of righting reflex when challenged with 35 mg/kg of leptazol (Table 2).

Table 2. *Seizure susceptibility in* p-*chlorophenylalanine* (*p-CPA*)*-treated rats.* Leptazol was reduced to 35 mg/kg for better evaluation of the *p*-CPA effect. Rats treated for 3 days with 100 mg/kg daily of *p*-CPA show a decrease in seizure threshold with 65% in Phase III, severe convulsions. Susceptibility to leptazol is markedly enhanced after a single strong dose of *p*-CPA as seen by 100% Phase III, severe convulsions, versus 70% leptazol controls Phase I and II. *P* values as in Table 1.

	Dose mg/kg	Phase I		Phase II		Phase III		Phase IV
		A	B	C	D	E	F	G
p-CPA‡	316	—	—	—	—	—	100 (20)	—
p-CPA†	3 × 100	20 (4)	—	—	15 (3)	25 (5)	40 (8)	—
Leptazol	35	45 (9)	—	25 (5)	—	36 (6)	—	—

The brain 5-HT after 316 mg/kg of *p*-CPA has been reported (Koe & Weissman, 1966) to decrease to 11% of control values as opposed to 21% of control levels following *p*-CPA for 3 days at 100 mg/kg.

p-CPA can reduce the shock intensity threshold for tonic extension in rats (Koe & Weissman, 1968) and also lower the resistance of mice to electroshock seizures (Chen, Ensor & Bohner, 1968). 5-HT, on the other hand, is reported (Laborit, Coirault & others, 1958) to protect animals subjected to convulsive fits induced by oxygen under high pressure. Moreover, many anticonvulsive compounds such as phenobarbitone, diphenylhydantoin, nitrozepam and others, have been shown to selectively increase 5-HT in animals in no other organ but the brain (Bonnycastle, Giarman & Paasonen, 1957).

Our experiments suggest that affecting brain 5-HT levels with 5-HTP in conjunction with a peripheral decarboxylase inhibitor, may result in decreased neuronal excitability induced by leptazol. It also seems that adequate penetration of 5-HTP into brain tissue cannot be achieved effectively without prior inhibition of peripheral decarboxylase activity (de la Torre, 1968). This is due to extracerebral decarboxylation and conversion of the injected 5-HTP to 5-HT which remains at the brain capillary level until breakdown by monoamine oxidase (de la Torre, 1970).

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Effects of cocaine and amphetamine on the metabolism of tryptophan and 5-hydroxytryptamine in mouse brain *in vivo*

The central stimulant drugs, cocaine and amphetamine, have both been shown to influence the metabolism of catecholamines in brain (Dengler, Spiegel & Titus, 1961; Baird & Lewis, 1964; Carlsson, Lindqvist & others, 1965; Corrodi, Fuxe & Hökfelt, 1967). Ross & Renyi (1969) found that cocaine is a potent inhibitor of 5-hydroxytryptamine (5-HT) uptake in brain slices *in vitro*, and Foote, Sheard & Aghajanian (1969) reported a stimulatory effect of amphetamine on midbrain raphe units, which presumably contain 5-HT. These results have prompted the present experiments on the effects of cocaine and amphetamine on rates of accumulation and disappearance of ^{3}H-5-HT in mouse brain *in vivo* after intravenous administration of [^{3}H]tryptophan.

Accumulation of ^{3}H-5-HT. When [^{3}H]tryptophan is administered to mice (male, NMRI, 18–22 g) by constant rate intravenous infusion, ^{3}H-5-HT accumulates in brain at an increasing rate (Schubert, Nyback & Sedvall, 1970a). Mice were pretreated with cocaine hydrochloride (30 mg/kg, i.p.) or (±)-amphetamine sulphate (15 mg/kg, i.p.). After 10 min [^{3}H]tryptophan (40 μCi/animal, 6·0 Ci/mmol) was infused during 20 min. Cocaine and possibly also amphetamine reduced the accumulation of ^{3}H-5-HT in brain in comparison to saline-treated animals (Table 1). No significant effect of the drugs on the contents of [^{3}H]tryptophan, endogenous 5-HT or tryptophan was found after this relatively short period following drug administration.

Table 1. *Effects of cocaine and amphetamine on levels of labelled and endogenous tryptophan and* 5-HT *in mouse brain after infusion of* [^{3}H]*tryptophan*, [^{3}H]tryptophan (40 μCi) was infused i.v. for 20 min starting 10 min after i.p. injection of saline, cocaine hydrochloride (30 mg/kg) or (±)-amphetamine sulphate (15 mg/kg). Animals were killed immediately after the infusion. Figures represent mean value ± s.e. from 6–8 animals.

Treatment	[^{3}H]tryptophan counts/min. 10^3/g	Tryptophan μg/g	^{3}H-5-HT counts/min. 10^3/g	5-HT μg/g
Saline	86 ± 6	4·1 ± 0·29	1·59 ± 0·14	0·29 ± 0·02
Cocaine	84 ± 5	4·3 ± 0·39	1·00 ± 0·10*	0·25 ± 0·01
Amphetamine	81 ± 9	4·0 ± 0·38	1·14 ± 0·15†	0·28 ± 0·02

* Differs from saline group ($P < 0·01$)
† Differs from saline group ($P < 0·05$)

Disappearance of ^{3}H-5-HT. After an intravenous injection of [^{3}H]tryptophan to mice, the 5-HT store in brain is maximally labelled within 30 min. Between 1–3 h after administration of the labelled precursor, ^{3}H-5-HT disappears from brain at a rate which appears to be exponential and is not altered by treatment with the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (Schubert & others, 1970a). Thus, in non-treated animals the disappearance of labelled 5-HT during the mentioned time interval is determined predominantly by the turnover rate of the amine. Saline, cocaine hydrochloride (30 mg/kg) or (±)-amphetamine sulphate (15 mg/kg) were administered intraperitoneally 1 and 2 h after the intravenous injection of [^{3}H]tryptophan (40 μCi/animal). Groups of animals were killed 1 or 3 h after precursor administration and the contents in brain of endogenous and labelled tryptophan and 5-HT were determined. The rates of disappearance of [^{3}H]tryptophan and ^{3}H-5-HT were significantly retarded by both drugs (Table 2). Cocaine caused a slight, but amphetamine a threefold increase of endogenous tryptophan content in brain. The level of endogenous 5-HT was possibly increased after amphetamine.

These experiments demonstrate that cocaine and amphetamine have effects on

Table 2. *Effects of cocaine and amphetamine on levels of labelled and endogenous tryptophan and* 5-HT *in mouse brain after injection of* [^{3}H]*tryptophan.* Saline, cocaine hydrochloride (30 mg/kg) or (±)-amphetamine sulphate (15 mg/kg) were administered i.p. 1 and 2 h after i.v. injection of [^{3}H]-tryptophan (40 μCi). Animals were killed 3 h after [^{3}H]tryptophan administration. Figures represent mean value ± s.e. from 8–9 animals.

Treatment	Time h	[^{3}H]tryptophan counts/min. 10^3/g	Tryptophan μg/g	^{3}H-5-HT counts/min. 10^3/g	5-HT μg/g
—	1	6·2 ± 0·4	4·8 ± 0·37	1·01 ± 0·07	0·39 ± 0·02
Saline ..	3	2·6 ± 0·2	4·1 ± 0·25	0·39 ± 0·02	0·38 ± 0·02
Cocaine ..	3	3·7 ± 0·2*	6·3 ± 0·33*	0·58 ± 0·03*	0·45 ± 0·02
Amphetamine	3	6·6 ± 0·4*	14·4 ± 1·03*	0·56 ± 0·03*	0·46 ± 0·02†

* Differs from saline group ($P < 0·001$)
† Differs from saline group ($P < 0·05$)

tryptophan and 5-HT metabolism in brain and display certain similarities. Both caused a significant increase of endogenous tryptophan levels in brain and retarded the rate of [^{3}H]tryptophan disappearance. This effect on tryptophan metabolism, which was much more pronounced for amphetamine than for cocaine, developed slowly. It was thus not significant in the accumulation experiment, i.e. 30 min after drug administration, but was prominent after 2 h in the disappearance experiment. The mechanism for the effect could be explained by an inhibitory effect of the drugs or their metabolites on tryptophan catabolism or binding, or both.

Cocaine, and possibly amphetamine decelerated rates of accumulation and disappearance of ^{3}H-5-HT in brain which could indicate a retardation of endogenous 5-HT synthesis and turnover rates. However, the effect of the drugs on 5-HT disappearance could be secondary in part to the drug-induced changes of tryptophan metabolism. Thus, the increased [^{3}H]tryptophan levels after cocaine and, above all, amphetamine treatment should accelerate ^{3}H-5-HT resynthesis, resulting in retardation of the ^{3}H-5-HT disappearance rate. However, it would seem difficult to explain the immediate inhibitory effect of the drugs on ^{3}H-5-HT accumulation as secondary to the slow but substantial increase of brain tryptophan levels. Therefore it is suggested from the present data that cocaine, and possibly also amphetamine, have effects on brain 5-HT metabolism which are partly secondary to changes of tryptophan metabolism, and partly direct. Cocaine is a potent inhibitor of *in vitro* 5-HT uptake in brain slices (Ross & Renyi, 1969), whereas amphetamine has no such effect (Pletscher & Bartolini, 1967). Both drugs are weak inhibitors of monoamine oxidase. The effect of cocaine on ^{3}H-5-HT metabolism is similar to that previously found for dimethylated tricyclic antidepressants (Schubert, Nybäck & Sedvall, 1970b), which also inhibit *in vitro* 5-HT uptake (Ross & Renyi, 1969). The influence of cocaine on 5-HT metabolism in brain may be interpreted as follows: reduction of 5-HT uptake or catabolism, or both, results in increased activation of 5-HT receptors. By a hypothetical negative feed-back mechanism, impulse activity and transmitter synthesis in 5-HT neurons are subsequently decelerated.

Regarding amphetamine our experiments demonstrate that this drug not only affects catecholamines but also influences transmitter metabolism in brain 5-HT neurons. The results could be explained by a decelerating effect of amphetamine on 5-HT synthesis, an effect which is difficult to relate to the stimulatory effect of the drug on activity of raphe units reported by Foote & others (1969). Since Glowinski, Axelrod & Iversen (1966) have presented evidence that the monoamine oxidase activity of tissues is reduced after treatment with amphetamine, at least some of the effects of amphetamine might follow from inhibition of monoamine oxidase activity.

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Restoration of blood pressure and heart rate responses to tyramine by infusion of 5-hydroxytryptamine in reserpine-treated pithed rats

In the pithed rat, pretreatment with reserpine abolishes the cardiovascular effects of tyramine due to depletion of intraneuronal stores of noradrenaline (Burn & Rand, 1960; Torchiana, Wenger & others, 1966; Clarke & Leach, 1968; Clarke, 1970). An infusion of noradrenaline or one of its precursors, by repleting the tissue stores, restores responses to tyramine (Burn & Rand, 1960; Torchiana & others, 1966). This is easier to demonstrate if deamination of the infused (or formed) amine is prevented by prior injection of drugs possessing monoamine oxidase activity (Clarke & Leach, 1968).

In the pithed reserpinized rat, there is a tissue uptake process for low doses of 5-hydroxytryptamine (5-HT) (Fozard, 1969) similar to that described previously for noradrenaline (Muscholl, 1961; Weiner & Trendelenburg, 1962; Van Zwieten, Widhalm & Hertting, 1965). It was tentatively suggested that 5-HT and noradrenaline shared a common uptake pathway into the sympathetic nerves (Fozard, 1969). The ability of infusions of 5-HT to restore responses to tyramine in reserpinized pithed rats would support such a suggestion.

A total of 21 female Wistar rats weighing 190–230 g were used. Those pretreated with reserpine were given 5 mg/kg intraperitoneally 18–22 h before the experiment. Rats were pithed under pentobarbitone anaesthesia and set up for femoral intravenous injection and recording of carotid blood pressure (Clarke & Leach, 1968). In most experiments heart rate was also recorded (Clarke, Hiscoe & others, 1966). Drugs, dissolved in saline, were given in volumes of 0·1 ml and washed into the animal with 0·2 ml of saline. Infusions were administered into a femoral vein by a Palmer slow injection apparatus at a rate of 2·5 ml/20 min. Test doses of tyramine were not given until 30 min after the heart rate had returned to pre-infusion levels.

The blood pressure and heart rate responses to a 25 μg dose of tyramine were abolished after pretreatment with reserpine (Fig. 1). Infusions of 5-HT (0·5 mg/kg in 20 min) routinely caused an increase in both blood pressure and heart rate, but failed to restore the response to tyramine when this was injected 30 and 60 min after the end

of the infusion (Fig. 1A). Higher doses of 5-HT could not be used since they caused rapid deterioration of the preparations. If an injection of either bretylium (1 mg/kg) or nialamide (20 mg/kg) was given intravenously 10 min before the infusion of 5-HT, the tyramine response was restored when tested 30 and 60 min after completion of the infusion. This is illustrated for bretylium in Fig. 1B. In confirmation of earlier observations (Clarke & Leach, 1968), the same dose of bretylium or nialamide given alone failed to restore the responses to tyramine.

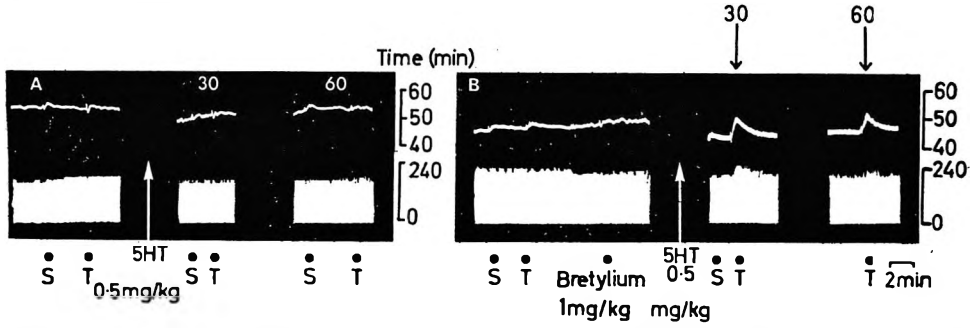

FIG. 1. Reserpine-treated pithed rats. Blood pressure (upper tracing; mmHg) and heart rate (lower tracing; beats/min) responses to tyramine 25 μg (T). A = Before and 30 and 60 min after an intravenous infusion of 5-HT (0·5 mg/kg in 20 min) at arrow. B = As for A except that bretylium (1 mg/kg) was injected 10 min before the infusion. S = Injection of 0·3 ml saline. Time 2 min.

The restored response to tyramine in the presence of bretylium showed quite rapid tachyphylaxis, being reduced to pre-infusion levels between 90 and 120 min after the infusion. Injection of desipramine (0·1 mg/kg) abolished the restored response to tyramine, had no detectable effects on responses to injected 5-HT, and enhanced responses to noradrenaline. In contrast, injection of bromolysergide (0·04 mg/kg) abolished both the restored responses to tyramine and the pressor response to injected 5-HT (Fig. 2). In preparations not treated with reserpine, bromolysergide had no detectable effects on responses to noradrenaline or tyramine in doses up to 0·5 mg/kg.

The temporary restoration of tyramine responses in reserpinized tissues by exposure to noradrenaline, its precursors, or their α-methylated equivalents, is suggested to be due to refilling of transmitter stores (for references, see Iversen, 1967). In our experiments the restored responses to tyramine appear to be mediated indirectly, since they

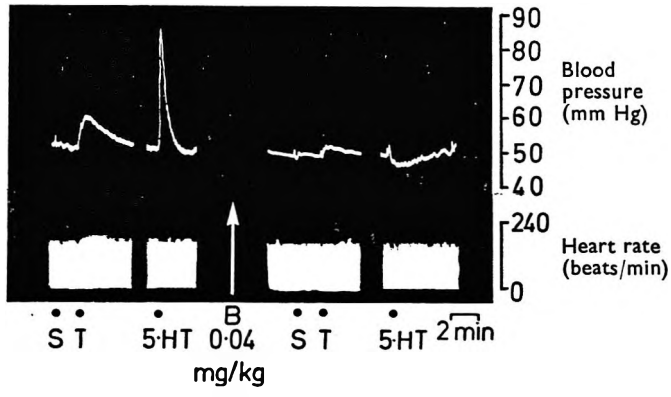

FIG. 2. Reserpine-treated pithed rat injected with bretylium (1 mg/kg) and given an infusion of 5-HT (0·5 mg/kg in 20 min) as in FIG. 1B. Blood pressure and heart rate responses to tyramine 25μg (T) and 5-HT 0·4μg (5-HT) before and after an intravenous injection of bromolysergide (0·04 mg/kg). Other details as in Fig. 1.

were antagonized by desipramine in doses which did not antagonize responses to either 5-HT or noradrenaline. Further, the mediator is likely to be 5-HT since the tryptamine D-receptor antagonist, bromolysergide, antagonized responses to 5-HT and responses to tyramine restored by 5-HT, but not responses to noradrenaline or tyramine mediated by noradrenaline.

The infusion dose of 5-HT used, only restored responses to tyramine when preceded by injection of drugs possessing monoamine oxidase activity. The results are analogous to those obtained by Clarke & Leach (1968), who demonstrated that pretreatment of reserpinized pithed rats with nialamide (20 mg/kg) or bretylium (1mg/kg) increased the efficacy of infusions of noradrenaline in restoring responses to tyramine. After bretylium, they observed an increased tissue retention of noradrenaline as a result of monoamine oxidase inhibition. The major route of inactivation of parenterally administered 5-HT in the reserpine-treated rat is also by oxidative deamination (Erspamer, 1956; Airaksinen, 1963; Axelrod & Inscoe, 1963). This occurs extremely rapidly after intravenous infusions (Fozard, 1969), and would explain the failure of infusions of 5-HT to restore responses to tyramine in untreated reserpinized preparations. Conversely, after nialamide or bretylium, deamination would be slowed and the restorative effects of 5-HT would be enhanced. In this connection, it has been shown that the tissue retention of [^{14}C]-5-HT after intravenous infusion in the pithed reserpinized rat was increased by prior injection of monoamine oxidase inhibitors, including bretylium (Fozard, 1969). The results would also indicate that 5-HT is penetrating to an intracellular site since monoamine oxidase is not located extracellularly (Blaschko, 1952; Kopin, 1964; Carlsson, 1965).

In conclusion these observations provide evidence for an uptake of 5-HT into an intracellular storage site accessible to tyramine. By analogy with similar results from experiments with noradrenaline (Clarke & Leach, 1968) this is likely to be the transmitter store within the sympathetic nerve endings.

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Effects of drugs on human foetal intestine: a preliminary investigation

In an attempt to study the pharmacological aspects of the development of smooth muscle we have investigated the effects of drugs on the isolated small intestine of the human foetus. After hysterotomy, the gastrointestinal tract was removed from foetuses of gestational age between 11 and 25 weeks, and stored in Krebs solution at 4°. Within 4–17 h after removal, a short length (2–3 cm) of small intestine was mounted in a 10 ml organ bath containing Krebs solution at 37° and the tone and movements of the longitudinal muscle recorded with an isometric transducer under a tension of 1–2 g. The tissue was allowed to equilibrate for 2 h before the application of drugs. Drug concentrations are expressed as the final bath concentration of base in g/ml.

Segments of small intestine from over 70 foetuses have been examined and most of these showed spontaneous activity throughout the experiment. All tissues contracted in the presence of acetylcholine in concentrations between 10^{-8} and 10^{-6}; the sensitivity did not appear to be related to gestational age but the more mature tissue was capable of developing a greater tension. The response to acetylcholine was potentiated by eserine (4×10^{-8}) and inhibited by atropine (4×10^{-8}), but not by hexamethonium (3×10^{-6}) suggesting that acetylcholine was acting on muscarinic receptors.

Histamine (2×10^{-8}–2×10^{-6}) caused contractions only in 9 out of 12 tissues and a biphasic response, relaxation followed by contraction, in the others. The histamine response appeared to be dependent on the maturity of the foetus because the biphasic response was observed only in tissues from foetuses of gestational age of 20 weeks or more. In the oldest tissue studied, from a 25-week foetus, histamine (4×10^{-8}) produced a relaxation, but later in the experiment a biphasic response was obtained. Neither the relaxation nor contraction was blocked by hexamethonium (5×10^{-6}) but both were blocked by mepyramine (10^{-6}).

The response to 5-hydroxytryptamine (5-HT) (2×10^{-8}–4×10^{-7}) also appeared to be dependent upon the age of the tissue. The longitudinal muscle from foetuses aged between 14 and 18 weeks contracted to this drug whilst a biphasic response of relaxation and contraction was observed in tissue from six 20-week foetuses. Responses to 5-HT were unaffected by hexamethonium (5×10^{-6}).

Noradrenaline (10^{-8}) (Fig. 1), phenylephrine (10^{-5}), adrenaline (10^{-7}) and isoprenaline (10^{-6}) inhibited spontaneous activity and caused relaxation in all the smooth muscle preparations of foetal intestine examined. The response to noradrenaline was blocked completely by propranolol (10^{-6}), reduced by phentolamine (10^{-5}) and unaffected by hexamethonium (5×10^{-6}) or guanethidine (10^{-5}) (Fig. 1).

The actions of nicotine, and the nicotine-like ganglionic stimulant dimethylphenylpiperazinium, were studied on most preparations; their effects were qualitatively similar. Low concentrations (6×10^{-7}) of nicotine usually produced a relaxation which was blocked by hexamethonium (5×10^{-6}), guanethidine (10^{-5}) (Fig. 1) or propranolol (10^{-6}) but in about 10% of the tissues a contraction was elicited which was blocked by atropine (4×10^{-8}). With higher doses of nicotine (5×10^{-5}), the longitudinal muscle relaxed and then contracted whilst, with doses greater than 10^{-4}, only a contraction was observed; the nicotine-induced contraction was unaffected by hexamethonium (5×10^{-6}), atropine (4×10^{-7}) and mepyramine (10^{-6}). The relaxation obtained with a low dose of nicotine (10^{-6}) could be reduced by the prior application of a higher dose (5×10^{-5}) of the drug. The duration of a response to nicotine appeared to be dependent on gestational age. In muscle from young foetuses

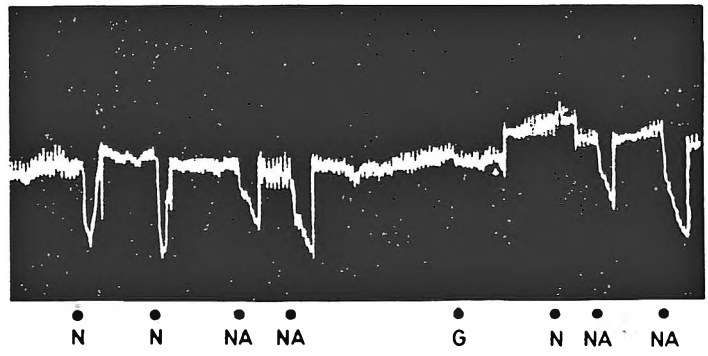

FIG. 1. Small intestine from 20-week foetus. Responses to nicotine (N) 6×10^{-6}, $1{\cdot}2 \times 10^{-5}$ and noradrenaline (NA) 5×10^{-8}, 10^{-7} before the administration of guanethidine (G) 10^{-5} for 8 min. After the guanethidine, the response to nicotine (N) 6×10^{-6} has been blocked whilst the responses to noradrenaline (NA) are unaltered. The relaxation to the first dose of nicotine is equivalent to a change in tension of 0·5 g.

(11–14 weeks) the offset of the effect was slow and unless an interval of 20 min elapsed between successive doses of the same amount of nicotine the response decreased. By contrast, in tissue from more mature foetuses (e.g. 20 weeks) consistent responses could be obtained by adding the drug at 6 min intervals.

In this study we have shown that longitudinal muscle from human foetal small intestine is capable of responding to acetylcholine, amines and ganglion-stimulants and that there may be a change in the response to histamine and 5-hydroxytryptamine at about 20 weeks. This extends the work of Boreus (1967, 1968) who reported that the muscarinic receptor is fully developed in the ileum of the 12-week human foetus.

It is of interest to compare the present results with those of other authors obtained with adult human small intestine. Thus, Bennett (1965) reported that histamine most frequently produced a relaxation but that contractions and biphasic responses were also seen; in the foetal tissue the tendency was for muscle from 14–18 week foetuses to contract whilst that from more mature foetuses gave a biphasic response. In adult tissue, 5-HT produced a contraction (Fishlock, 1964; Bennett, 1965; Whitney, 1965) as in tissue from younger foetuses but, with muscle from older foetuses, a biphasic response was seen. The relaxation to low doses of nicotine by the foetal muscle corresponds with the response of adult tissue (Bennett, 1965; Fishlock & Parks, 1966). This relaxation could be prevented by the presence of a ganglion blocker, a post-ganglionic sympathetic nerve blocker or a β-adrenoceptor blocker which suggests that the response involves release of an adrenergic mediator. This contrasts with the suggestion of Burn (1968) that the sympathetic innervation of the rabbit intestine changes from cholinergic to adrenergic between the third and eighth day after birth.

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Relation between drug elimination kinetics in intact animals and isolated perfused liver systems: phenylbutazone

Recent studies have shown the usefulness of the isolated perfused liver for investigations of the effects of enzyme induction and of distribution of drug on the drug's metabolism (von Bahr, Alexanderson & others, 1970; Nagashima, Levy & Sarcione, 1968; Levy & Nagashima, 1969). If a drug is eliminated only by biotransformation in the liver, good agreement between its *in vitro* and *in vivo* elimination rate constants may be obtained by correcting for the difference in drug distribution (liver: extrahepatic sites) in the perfused liver system and in the intact animal (Nagashima & others, 1968). This approach has been used successfully with bishydroxycoumarin and has yielded similar "true" elimination rate constants *in vitro* and *in vivo* even though the "apparent" elimination rate constants in perfused rat liver systems were three to four times higher than in the intact animals (Nagashima & others, 1968). The results to be reported here show that there is also a good quantitative correlation between *in vitro* and *in vivo* elimination rate constants in animals pretreated for various lengths of time with phenobarbitone, a potent microsomal enzyme inducing agent.

The elimination rate constant of phenylbutazone was determined in male Sprague-Dawley rats weighing 200 to 300 g and in isolated perfused livers obtained from similar animals. The intravenous dose (50 mg/kg) and the amount of drug in the

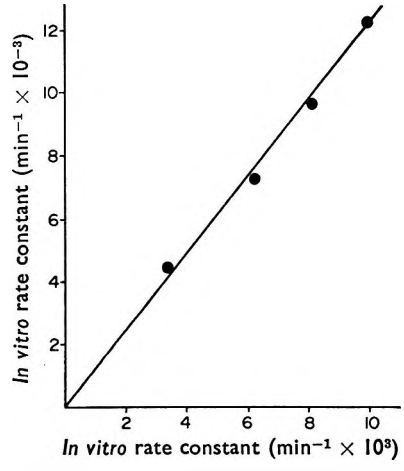

FIG. 1. Relation between *in vitro* and *in vivo* elimination rate constants of phenylbutazone in isolated perfused liver and in intact rats, respectively. From left to right, the data points represent values from control animals and from animals pretreated for 1, 2, and 3 days with phenobarbitone. The results are calculated from the experiments shown in Table 3 in the work of von Bahr & others. The points represent the mean values in these experiments.

perfusion system (5 mg in about 100 ml diluted blood) yielded similar drug concentrations in the plasma. Other details of the experiments are reported elsewhere (von Bahr & others, 1970). Some of the animals were pretreated for 1, 2, or 3 days with daily intraperitoneal doses of phenobarbitone, 80 mg/kg.

Pretreatment with phenobarbitone increased the elimination rate constant of phenylbutazone up to three-fold, depending on the length of the pretreatment. Fig. 1 shows that there is a good correlation between the *in vitro* and *in vivo* elimination rate constants. The average ratio of these constants, *in vitro*: *in vivo*, is 1·24. This ratio depends of course on experimental conditions, such as the volume and composition of the perfusion fluid and (sometimes) the perfusion rate (Nagashima & others, 1968; Nagashima & Levy, 1968).

The experimental results now available show that effects on drug metabolism due to changes in drug distribution, resulting from changes in plasma protein concentration, and drug metabolizing activity, due to enzyme induction, are reflected to the same degree in intact animals and in isolated perfused liver systems in the case of drugs, like phenylbutazone and bishydroxycoumarin, which are eliminated solely by biotransformation in the liver. If the animal excretes part of the drug in an unchanged form, similar correlations should be possible by using the *in vivo* drug biotransformation rate constant rather than the elimination rate constant. These findings encourage further use of the isolated perfused liver for drug metabolism studies.

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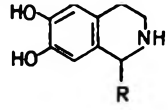
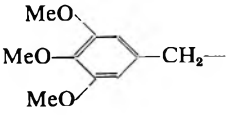
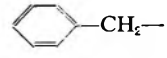
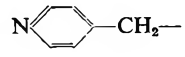
Influence of substituted tetrahydroisoquinolines on lipolysis, *in vitro*

The important structural requirements involved in the mobilization of free fatty acids (FFA) in adipose tissue induced by adrenergic agents, particularly noradrenaline, isoprenaline, and adrenaline include a catechol nucleus, an alcoholic hydroxyl group on the β-carbon atom, and bulkyl alkyl or arylalkyl functions on the amino-nitrogen of the ethylamino side-chain (Feller & Finger, 1966; 1970; Wenke, 1966; Cernohorsky, Cepelik & others, 1966). While it is well-known that the adrenergic activity of substituted phenethylamines is greatly enhanced by *N*-substitution, few studies have been reported on the influence of tetrahydroisoquinoline (THI) derivatives in adipose tissue receptor systems. Holtz, Stock & Westermann (1964) found the isoquinoline derivative, 3,4 dihydroxybenzyl-6,7 dihydroxy-THI, to be 6 times less active than noradrenaline in the mobilization of FFA, *in vivo* and *in vitro*. In other studies (Iwasawa & Kiyomoto, 1967; Yamato, Hirakura & Sugasawa, 1966) it was reported that several THI analogues were more active than adrenaline on tracheal relaxation. Of the THI's tested 1-(3′,4′,5′-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-THI was found to be the most potent. Since tracheal relaxation and lipolysis are considered to be β-adrenergic receptor systems (Wenke & others, 1966), it was of interest to test additional THI derivatives for their ability to release glycerol from adipose tissue, *in vitro*.

Epididymal fat pads from 4–6 male Harlan-Wistar rats (200–250 g) per experiment were transferred to Krebs Ringer bicarbonate buffer, pH 7·4 and minced with scissors to yield adipose tissue fragments. Various concentrations (10^{-3} — 10^{-9}M) of each agonist and 300 mg of adipose tissue were incubated at 37° for 60 min in 2·5 ml of bicarbonate buffer which contained 4% bovine serum albumin (Finger, Page & Feller, 1966). After 60 min, the reaction was terminated by the addition of 1 ml of trichloroacetic acid (10%). The rate of lipolysis was determined from the glycerol formed, by oxidation according to Lambert & Neish (1950) and assay of the resulting formaldehyde by the method of Nash (1953). A maximal release of glycerol calculated to be 5·4 μmol glycerol/g tissue h^{-1} was observed for all agonists with the exception of dopamine. This value was then used to calculate the percent response of adipose tissue to varying concentrations of agonist to obtain dose-response relations. Experiments were repeated at least 4 times to establish dose-response curves to permit the calculation of the intrinsic activity and affinity (pD_2 values) constants as described by Miller, Becker & Tainter (1948) and Ariens & Simonis (1962). From a comparison of the pD_2 values shown in Table 1, the rank order of activity for the compounds was THI (I) > noradrenaline > THI (II) $\geqslant$ THI (III) >> dopamine. The trimethoxy-THI analogue (I) was the most active derivative tested and was found to be about 5 times more potent than noradrenaline. Although the 1-benzyl (II) and 1-(4′-pyridylmethyl) (III)- THI's were less active than noradrenaline, they were much more effective than dopamine. The difference in pD_2 values observed between noradrenaline and dopamine is attributed to the presence or absence of the β-hydroxyl group respectively. While this chemical moiety has been ascribed an important role for the catecholamine-induced mobilization of FFA; the THI derivatives which lack the β-hydroxyl group retain potent lipolytic activity in this adrenergic adipose tissue system. We have also observed that the β-blocker, propranolol, is a competitive inhibitor of the lipolysis induced by noradrenaline and the 3,4,5-trimethoxy compound (I). In the presence of 10^{-5}M propranolol, a parallel shift of 1·5 log dose units was obtained for both of these compounds (see Table 1). This evidence suggests that the THI derivatives may interact at the same adrenergic receptor system in adipose tissue as noradrenaline. Coupled with the previous study on the effects of

Table 1. *Intrinsic activity and affinity (pD_2 values) constants for substituted tetrahydroisoquinolines* (THI) *on the release of glycerol from adipose tissue,* in vitro.



No.	Compound	R	$pD_2 \pm$ s.d.	Intrinsic activity
I	1-(3′,4′,5′-Trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-THI		7·2 ± 0·4	1·0
	1-(3′,4′,5′-Trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-THI & 10^{-5}M (±)-propranolol		5·7 ± 0·3	1·0
	(—)-Noradrenaline		6·5 ± 0·3	1·0
	(—)-Noradrenaline & 10^{-6}M (±)-propranolol		4·9 ± 0·1	1·0
II	1-Benzyl-6,7-dihydroxy-1,2,3,4-THI		5·8	1·0
III	1-(4′-Pyridylmethyl)-6,7-dihydroxy-1,2,3,4-THI		5·6	1·0
	Dopamine		3·9	0·3

THI compounds on tracheal relaxation, our results clearly indicate that appropriately substituted THI's are potent β-adrenergic agonists.

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Transfer of morphine tolerance in rats by brain extracts

Ungar & Cohen (1966) first described the transfer of tolerance to mice with injections of brain extracts from morphine tolerant animals. Other investigators (Tirri, 1967; Smiths & Takemori, 1968; Cox & Ginsburg, 1969) were unable to transfer tolerance in this way. We now report the transfer of tolerance from rats to rats.

Thirty white male rats, 155–250 g, had morphine hydrochloride morning and night intraperitoneally; the starting dose was 20 mg/kg twice daily. On 3rd, 6th, 9th, 12th, 15th and 18th days, the dose was increased by daily increments of 40 mg/kg so that on the 18th day the daily dose was 280 mg/kg. This dose was maintained until the 23rd day. Ten control rats received equivalent volumes of saline by the same route every morning and night. On the 23rd day two rats from the control group and six rats from the morphine-treated group were given 25 mg/kg of nalorphine hydrochloride intraperitoneally and showed obvious signs of the abstinence syndrome (Kaymakçalan & Woods, 1956).

On day 24, 16 h after the last dose of morphine, 10 rats were decapitated and their brains transferred immediately to 75 ml of cold acetone and homogenized with a Böhler homogenizer for 5 min at 15 000–17 000 rev/min, the homogenizer being cooled externally with ice cold water. The volume of the extract was made to 150 ml with acetone and centrifuged (Sorvall RC2B) for 2 h at 39 000 *g* and −2 to −5°. After 18–24 h at −2° the supernatant was discarded and the precipitate mixed with 50 ml saline and homogenized again at 15 000–17 000 rev/min for 3 min.

Intact rats received intraperitoneally 0·8–1·0 ml of the brain extract, either obtained from the rats rendered tolerant to morphine or from the saline treated group, and their pain reaction time tested with the hot plate (57·5 ± 0·5°) method (Johannesson & Woods, 1964), before and 24 h after injection of the extract. After second testing they received morphine (6 mg/kg i.p.) and 90 min later were tested again.

We observed an obvious tolerance to the analgesic effect of morphine in animals treated with the brain extract from tolerant animals (Table 1), results which are in accordance with those of Ungar & Cohen (1966).

Table 1. *Reaction time to thermal stimuli* (seconds ± s.e.)

Group	First testing: Control (before injection)	Second testing: 24 h after injection of brain extract and 1st testing	Third testing: 90 min after injection of morphine, 6 mg/kg, and 2nd testing
Control	7·33 ± 0·56 n = 6	—	13·1 ± 1·01* n = 6
Normal rats brain extract injected group	8·0 ± 0·18 n = 6	8·0 ± 0·43 n = 6	12·8 ± 1·44† n = 6
Tolerant rats brain extract injected group	7·9 ± 0·45 n = 10	9·2 ± 0·85 n = 10	9·6 ± 0·60‡ n = 10

* $P < 0{\cdot}0005$ when compared with initial value of same group.
‡ $P < 0{\cdot}0025$ when compared with initial value of same group.
† Non-significant when compared with initial value of same group.

Inhibition of protein synthesis can prevent the development of tolerance (Cohen, Keats & others; 1965; Loh, Way & Shen, 1969; Cox & Osman, 1970); it is reasonable to assume the transfer of material of a protein nature. Our experiments shed no light on the precise actions of the transferred material.

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Ultrastructural changes induced by pregnenolone nitrile in the rat liver

In rats, intoxication with indomethacin, digitoxin (Selye, 1970a), cyclophosphamide (Selye, 1970b) and various other drugs can be prevented by pregnenolone nitrile (3β-hydroxy-20-oxo-5-pregnene-16α-carbonitrile). So far, this compound has been found to be the most active inhibitor of intoxication among over 300 steroids tested in our laboratory. Its ability to cause liver hypertrophy and increased pentobarbitone clearance in the blood is consistent with the view that this steroid nitrile acts through microsomal enzyme induction (Selye, 1970a,b). Therefore, it seemed of interest to determine whether this compound causes ultrastructural alterations in the hepatocytes.

Female ARS/Sprague-Dawley rats (Madison, Wisconsin, U.S.A.) of average weight 100 g and maintained on freely available Purina Laboratory Chow and tap water were used. Pregnenolone nitrile (10 mg in 1·0 ml of water) was administered orally, by soft rubber catheter, twice daily for 5 days. The animals were killed by destruction of the medulla oblongata on the 6th day, 16 h after the last gavage. A section of the liver was excised, minced, fixed in Millonig's osmium solution and processed for electron microscopic studies, as described elsewhere (Kovacs, Blascheck & Gardell, 1970; Gardell, Blascheck & Kovacs, 1970).

Pregnenolone nitrile-treated animals exhibited a marked proliferation of the smooth-surfaced, with a relative decrease of the rough-surfaced, endoplasmic reticulum (Fig. 1A and B). It could not be ascertained whether this increase was due to degranulation and transformation of the rough-surfaced into the smooth-surfaced endoplasmic reticulum, or whether it represented *de novo* synthesis. Long, smooth-surfaced lamellae were seen in some places; these were the originally ribosome-studded membranes which had undergone degranulation. The mitochondria were somewhat swollen and, in some cases, they assumed a homogeneous appearance with the disappearance of the cristae. Lipid content increased moderately, and the microvilli in the bile canaliculi seemed to be hypertrophied.

Proliferation of the smooth-surfaced endoplasmic reticulum is not a specific effect of pregnenolone nitrile; it is also induced by various other compounds, such as phenobarbitone (Fouts & Rogers, 1965), tolbutamide (Remmer & Merker, 1965), spironolactone (Kovacs & others, 1970), norbolethone (Gardell & others, 1970) and certain other steroids (Horvath, Kovacs & others, 1970). It has been assumed that this change indicates activation of various microsomal drug-metabolizing enzymes in the liver (Fouts & Rogers, 1965; Conney, 1967). However, further investigations are required to clarify the pathophysiological significance of smooth-surfaced reticulum hypertrophy and of the role played by pregnenolone nitrile in causing proliferation of smooth membranes.

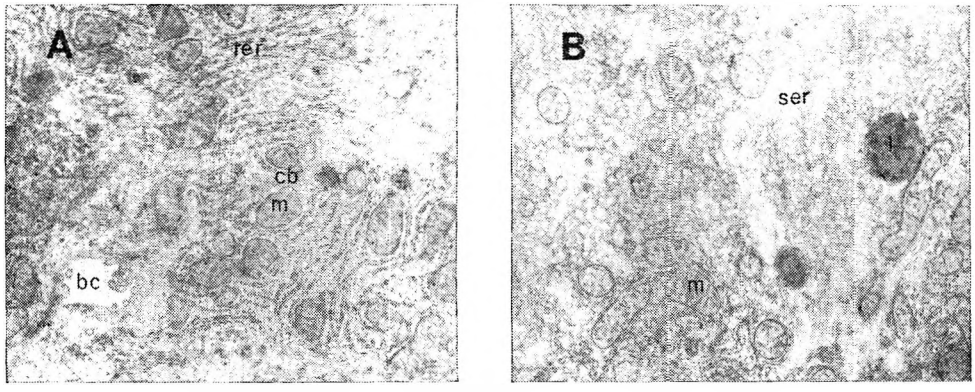

FIG. 1.A Untreated rat. Portions of two hepatocytes showing characteristic features, r e r: rough-surfaced endoplasmic reticulum; m: mitochondrion; b c: biliary canaliculus; c b: cell border; × 13,300.

B. Pregnenolone-treated rat. Accumulation of smooth-surfaced endoplasmic reticulum can be seen in a portion of hepatocyte, s e r: smooth-surfaced endoplasmic reticulum; m: mitochondrion; l: lipid; × 12 000.

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Stereotyped behaviour induced by (−)-ephedrine in rats

Amphetamine induces stereotyped behaviour in rats which is characterized by the decrease of locomotor activity, compulsive continuous movements of head, sniffing, licking and biting (Randrup, Munkvad & Udsen, 1963; Randrup & Munkvad, 1969; Ernst, 1967, 1969). The stereotyped behaviour has also been described in mice, guinea-pigs, cats, monkeys, and in man (Randrup & Munkvad, 1967). The behaviour is seen also with substances chemically related to amphetamine and with substances that are not—like methamphetamine, phenmetrazine, pemoline, apomorphine, cocaine, and tryptamine (Fog, 1969; Randrup & Munkvad, 1969).

There appear to be no published reports implicating ephedrine in stereotyped behaviour, even though it is a β-hydroxylated derivative of methamphetamine. I now report the induction of stereotyped behaviour in rats by (−)-ephedrine.

Wistar rats of either sex, 180–200 g, were injected intraperitoneally with (−)-ephedrine, (BDH) 40–140 mg/kg. Groups of 5 rats were placed in wire cages (21 × 21 × 25 cm) for 4 h and stereotyped activity was scored every 0·5 h by a scoring system similar to that of Janssen, Niemegeers & others (1967).

In other experiments the influence of chlorpromazine (4 mg/kg), haloperidol (0·5 mg/kg), and L-α-methyldopa (400 mg/kg) on stereotypy induced by ephedrine and amphetamine was also scored. Chlorpromazine and haloperidol were injected 0·5 h, and L-α-methyldopa 3 h before (−)-ephedrine (100 mg/kg) or (±)-amphetamine (10 mg/kg).

Ephedrine, 40–80 mg/kg, induced only sniffing and non-continuous licking and biting. The sniffing was observed especially near the walls. With higher doses of ephedrine (100–140 mg/kg), the stereotyped behaviour was manifested by sniffing at the floor and continuous licking and biting. The same behaviour was observed after amphetamine 10 mg/kg. Ephedrine- and amphetamine-induced stereotypes were antagonized by chlorpromazine, haloperidol and L-α-methyldopa.

Agents producing abnormal behaviour may be divided into two groups; those acting directly like apomorphine, and those acting indirectly, presumably by the release of dopamine in the central nervous system, like amphetamine, (Ernst, 1967; Randrup & Munkvad, 1969; Maj & Przegaliński, 1967).

Thus, stereotypy induced by high doses of ephedrine 100–140 mg/kg resembles that produced by amphetamine both in behaviour and in the mechanism of its development.

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Some pharmacological actions of alytesin and bombesin

Alytesin(I) and bombesin(II) are two natural tetradecapeptides recently isolated from methanol extracts of the skin of three European amphibians belonging to the family Discoglossidae. Alytesin has been found in the skin of *Alytes obstetricans*, bombesin in the skin of *Bombina bombina* and *Bombina variegata variegata*. Bombesin or a bombesin-like peptide is present also in the skin of *Bombina variegata pachypus* (Anastasi, Erspamer & Bucci, 1970).

(I) Pyr-Gly-Arg-Leu-Gly-Thr-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

(II) Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

The two polypeptides differ only in two amino-acid residues, the second and the sixth from the *N*-terminus, which are glycine and threonine in alytesin and glutamine and asparagine in bombesin.

Bombesin and alytesin can be easily demonstrated on paper chromatograms or electropherograms of crude or, much better, semi-purified extracts by means of colour reactions: Pauly reaction (histidine), *p*-dimethylaminobenzaldehyde reaction (tryptophan), and Sakaguchi reaction (arginine). Alytesin and bombesin may be accompanied by other compounds giving similar colour reactions.

The spectrum of biological activity of alytesin and bombesin is characteristic, and the distinction of this polypeptide family from other families is quite easy by means of parallel bioassay. The pharmacological study of bombesin and alytesin is in progress. So far, the following effects may be considered as well established for alytesin.

There was a hypertensive action in the dog, with marked tachyphylaxis. The threshold dose was less than 1 μg/kg by rapid intravenous infusion but the effect declined on continued administration of the polypeptide. The effect on blood pressure remained unchanged after pretreatment with α-adrenergic blocking agents. There was no cross-tachyphylaxis with Val^5-angiotensin. Alytesin was approximately ten times less potent than Val^5-angiotensin in its intensity of action, but hypertension elicited by alytesin lasted much longer.

It had potent stimulant action on the rat oestrous uterus being usually 2–4 times more potent than bradykinin and at least as potent as synthetic oxytocin (Syntocinon). The threshold dose was about 0·01 ng/ml nutrient fluid and there was a fair dose-response relation. Tachyphylaxis was either absent or moderate. Large doses of alytesin often elicited a tonus increase persisting for hours, in spite of repeated washing with fresh nutrient solution. The effect was atropine-resistant.

There was intense stimulant action on the rat and the guinea-pig colon, as well as on the cat ileum. Tachyphylaxis was absent or moderate and there was again a satisfactory dose-response relation, especially for the guinea-pig colon and cat ileum. Threshold doses ranged between 0·03 and 0·3 ng/ml. Atropine had no appreciable effect. In contrast to the colon, the ileum of the guinea-pig was generally poorly sensitive to alytesin, which produced repeated spikes of contraction, something lasting for hours. Tachyphylaxis was evident and atropine greatly reduced or inhibited the response to the polypeptide.

Alytesin had remarkable stimulant action on the gastric secretion of the chicken and the dog. In the chicken the intravenous infusion of 10–25 ng/kg min^{-1} of alytesin elicited a 50–150% increase in flow of gastric juice and an increase in acid and pepsin outputs. The concentration of pepsin in gastric juice was nearly doubled. Alytesin possessed approximately 5 to 10% of the potency of caerulein. The effect was inhibited by atropine. In dogs provided with denervated gastric pouches alytesin manifested approximately 5% of the action of caerulein and again the secretagogue effect was inhibited by atropine.

By methods measuring transmucosal potential differences and short-circuit current it was shown that alytesin caused an increased transport of Cl^- ions from the serosal to the mucosal surface of the isolated gastric mucosa of *Rana esculenta* and *Bufo viridis*. The threshold concentration was approximately 0·05–0·1 μg/ml of bath fluid. Alytesin was at least as active as pentagastrin, but 500–1000 times less active than caerulein. The effect was vaguely proportional to the dose and atropine-resistant.

There was a moderate hyperglycaemic effect in anaesthetized dogs and rats. In the rat, 15 μg/kg of alytesin given subcutaneously produced a 70% increase of the blood sugar level lasting 2 h while 50 μg/kg produced a 90% increase lasting for more than 5 h. In the dog, the intravenous infusion of 1 μg/kg min^{-1} of alytesin, for 10 min, caused, in addition to hypertension, an increase in blood sugar levels from 120 to 200 mg %, a progressive increase of immunoreactive insulin levels in femoral artery blood (up to 300%) and a 25–30% reduction of blood calcium levels. Return to basal values took 10–30 min.

The following isolated preparations were insensitive or poorly sensitive to alytesin or showed marked tachyphylaxis (in parentheses are the threshold doses per ml of nutrient fluid): rabbit non-pregnant uterus (>0·1 μg/ml), cat post-partum uterus (>0·5 μg/ml); uteri of guinea-pigs and hamsters pretreated with oestrogens (>0·1 μg/ml); rabbit large intestine (0·1 ng/ml, but prompt and intense tachyphylaxis), rabbit duodenum (0·1 ng/ml, but intense tachyphylaxis; occasionally stimulation was preceded by short-lasting inhibition of tone and movements), rat duodenum (no stimulation up to 0·1 μg/ml, doubtful relaxation), chicken terminal ileum and rectal caecum (1–10 ng/ml, no dose-response relation); guinea-pig tracheal chain (>5 μg/ml). The cat, guinea-pig and hamster uteri responded to 2–20 μU oxytocin/ml. The *in situ* gall bladder of the guinea-pig was contracted by intravenous injections of 50–400 ng/kg of alytesin.

Bombesin presented a spectrum of biological activity indistinguishable from that of alytesin. On some preparations it appeared slightly less active. Several fragments of the alytesin and bombesin molecules have been prepared by synthesis or by enzymic hydrolysis, and are being examined.

Nakajima, Tanimura & Pisano (1970) have recently isolated from methanol extracts of the skin of the American frog *Rana pipiens* an endecapeptide, ranatensin, the chemical resemblance of which with alytesin and bombesin appears obvious from the structural formula:

Pyr-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2

In preliminary pharmacological investigation (Geller, Govier & others, 1970) ranatensin manifested an hypertensive effect in dogs and rabbits, but was hypotensive in rats and monkeys. Moreover it stimulated the rat isolated uterus and guinea-pig ileum, and aortic strips of the rabbit. It had no effect on rat aortic strips and relaxed the rat duodenum.

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The potentiating effects of ethanol on responses of aortic strips to stimulant drugs

The assumption has been made that ethanol itself has no discernible effects on smooth muscle contractility. In the experiments reported below, it was observed that as little as 0·05 ml of ethanol in a 15 ml muscle chamber (58 mM) enhanced the responses to a variety of agonists in aortic strips and in a higher concentration (348 mM) exerted direct contractile effects.

Rabbit aortic strips were prepared as described previously (Kalsner & Nickerson, 1968a) and suspended under 2 g tension in muscle chambers of 15 ml capacity. The bathing medium was Krebs-Henseleit solution (containing 0·03 mM disodium EDTA) which was maintained at 37°. Responses were recorded isotonically on a kymograph drum moving at 1·8 mm/min. Concentrations of noradrenaline bitartrate (Calbiochem), methoxamine hydrochloride (Burroughs Wellcome) and histamine dihydrochloride (Calbiochem) are referred to as the weight of the base in g/ml in the muscle chambers. Ethanol (absolute ethanol, Consolidated Alcohols, Toronto) and potassium chloride (British Drug Houses) are referred to in terms of molarity. Reserpine (Nutritional Biochemicals) was dissolved in 10% ascorbic acid and rabbits were injected intramuscularly with 1 mg/kg about 18–24 h before death. Mean values are reported with their standard errors.

In preliminary experiments it was found that 58, 116 and 174 mM ethanol progressively enhanced the amplitude of responses to a low concentration of noradrenaline (3×10^{-9} g/ml) without significantly increasing the resting tone of aortic strips (Fig. 1a). The potentiation was sustained during the period of exposure of strips to ethanol (usually 10 to 20 min) and was reproducible at 30 min intervals.

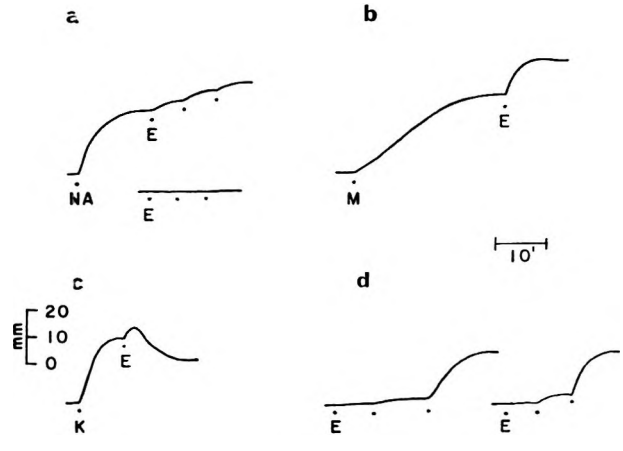

FIG. 1. Effects of ethanol on aortic strips. a. Upper trace shows effect of cumulative additions of ethanol (E) (58, 116 and 174 mM) on response to noradrenaline (NA) (3×10^{-9} g/ml); lower trace shows effect of ethanol alone. b. and c. Effects of ethanol (174 mM) on responses to methoxamine (M) (5×10^{-8} g/ml) and potassium (K) (20 mM). d. Responses of untreated (left) and reserpine-pretreated (right) strips to cumulative concentrations of ethanol (174, 348 and 696 mM).

In a more detailed set of experiments, strips of aorta were contracted by concentrations of noradrenaline, methoxamine, histamine and potassium, on the sharply rising portions of their dose-response curves, and after responses had reached stable plateau values they were exposed to ethanol (174 mM). The augmentation of responses to noradrenaline (3×10^{-9} g/ml), methoxamine (5×10^{-8} g/ml) and histamine (5×10^{-8}

g/ml) produced by ethanol was approximately equivalent to doubling the concentrations of the agonists in the muscle chambers (Table 1). In contrast, contractions induced by potassium (20 mM) were increased much less by ethanol, in terms of an equivalent concentration of potassium alone (Table 1), and the initial potentiation was followed by a depression of response amplitude. Typical traces obtained from several of the above experiments are shown in Fig. 1b and c. Pretreatment of aortic strips with reserpine, to deplete endogenous catecholamines, did not reduce the potentiating effects of ethanol. Ethanol alone in a concentration of 174 mM exerted a barely detectable effect on the tone of quiescent strips; an increase of 0·9 ± 0·1 mm in 17 strips.

Table 1. *Effects of ethanol on the amplitude of contractions produced by various agonists*

Agonist	Concn	No. of strips	Contraction amplitude (mm)	Ethanol increment		Final equiv. * concn of agonist
				mm	%	
Noradrenaline	3×10^{-8} g/ml	8	28·4 ± 3·9	9·8 ± 0·5	39·0 ± 5·0	$8{\cdot}4 \times 10^{-9}$ g/ml
Histamine	5×10^{-8} g/ml	10	6·4 ± 0·7	7·5 ± 0·5	126·8 ± 12·7	$9{\cdot}2 \times 10^{-8}$ g/ml
Methoxamine	5×10^{-8} g/ml	7	14·2 ± 3·8	15·1 ± 1·3	176·1 ± 44·9	$9{\cdot}5 \times 10^{-8}$ g/ml
Potassium	2×10^{-2} M	11	19·3 ± 2·5	6·2 ± 0·6	38·9 ± 6·5	$2{\cdot}3 \times 10^{-2}$ M

* Calculated from separately determined dose-response curves to each agonist as described previously (Kalsner & Nickerson, 1968b).
Ethanol was added to the muscle chambers after responses to agonists had reached plateau values.

In other experiments it was observed that higher concentrations of ethanol contracted aortic strips directly (Fig. 1d). Six strips responded to 348 and 696 mM ethanol with mean amplitudes of 2·7 ± 0·4 mm and 16·3 ± 1·6 mm. Contractions by ethanol could be obtained repeatedly in these strips at 30 min intervals. Neither pretreatment of rabbits with reserpine (Fig. 1d), nor treatment of strips with phenoxybenzamine (1×10^{-6} g/ml for 10 min) appeared to modify responses to ethanol. This concentration of phenoxybenzamine markedly reduced or eliminated responses to 1×10^{-6} g/ml of noradrenaline, histamine and 5-hydroxytryptamine.

To assess the role of extracellular and cellular bound calcium in the effects of ethanol, aortic strips were immersed for 30 min in a calcium-free Krebs solution containing disodium EDTA (0·1 mM). This procedure virtually eliminates extracellular and loosely bound calcium for contractions by agents such as potassium and materially reduces the tissue stores of tightly bound calcium utilized by compounds such as noradrenaline (Waugh, 1962; Hinke, 1965; Hudgins & Weiss, 1968). Under these conditions, responses to potassium (20–40 mM) were completely blocked and those to noradrenaline (3×10^{-9} g/ml) reduced to 12% of their amplitude in the standard Krebs solution. The potentiating effect of ethanol (174 mM) on responses to noradrenaline was proportionately the same as in the standard calcium solution; approximately equivalent to doubling the concentration of noradrenaline in the muscle chambers. The direct contractile effects of ethanol (696 mM) were decreased in calcium-free solution to about the same extent as were the responses to noradrenaline.

A possible explanation of the present results is that low concentrations of ethanol (58–174 mM) interfere with the rebinding of calcium released into the neighbourhood of the contractile filaments by stimulant drugs. This could adequately explain the potentiation of responses to all agonists tested, regardless of the source of calcium for contraction. Higher concentrations of ethanol (348–696 mM) appear to directly mobilize tightly bound calcium for contractions.

The depression of the amplitude of responses to potassium, which followed the initial potentiation may reflect an additional action of ethanol to impair the transmembrane flux of ions. Hurwitz, Battle & Weiss (1962) previously reported that ethanol depressed high potassium contractions in guinea-pig ileum. They suggested that ethanol impaired the inward movement of ionized calcium from membrane sites.

Gimeno, Gimeno & Webb (1962) observed that low concentrations of ethanol (24 to 192 mM) depressed the contractility of isolated rat atria and Fewings, Hannah & others (1966) found that ethanol administered into the brachial artery directly constricted the blood vessels of the forearm and hand. The present finding that as little as 58 mM ethanol enhanced responses to stimulant drugs in aortic strips indicates that it should be abandoned as a solvent in studies of the effects of water-insoluble drugs on smooth muscle reactivity. In addition, experiments in which it has been used should be re-interpreted with caution.

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Stress-induced increases in catecholamines in the brain of the young chick

The suggestion that stimulation of turnover of 5-hydroxytryptamine (5-HT) with sedation as the behavioural correlate (Hanig, Aiello & Seifter, 1970) prompted this short report. Several other investigators have implicated the catecholamines in sedation or depression, based upon direct intracerebral administration in subjects that have mature blood-brain barriers (Feldberg & Fleischhauer, 1965) and in studies with the newly hatched or young chick which, because of its lack of a blood-brain barrier to these biogenic amines (Waelsch, 1955; Lajtha, 1957; Key and Marley, 1962), becomes sedated upon their peripheral administration (Mandell & Spooner, 1968).

We have been studying the effects of reserpine, administered during embryogenesis of the domestic chicken, upon some biochemical and behavioural parameters post-natally (Sparber & Shideman, 1968, 1969a). The possibility that lower levels of brainstem catecholamines in chicks hatched from eggs injected with drug compared to eggs injected with vehicle might be due to an attenuated effect of stress by reserpine. If moderate stress could increase catecholaimine levels in the brain, perhaps disproportionately greater differences between two groups might result after more severe stress.

Six, twenty-three day-old male chickens hatched from eggs injected before incubation, with the vehicle in which reserpine was dissolved in an earlier study (Sparber & Shideman, 1968), were used. Three were subjected to a stressful situation which consisted of being placed within a box attached to a mechanical shaker (Eberbach Corp., Ann Arbor, Michigan) and shaken vigorously for 2 min. At the end of this time they were killed by decapitation and whole brain catecholamines or adrenaline and noradrenaline were determined by a semi-micro method (Sparber & Shideman, 1969b). The 3 chicks not stressed were taken directly from their brooder and killed.

The catecholamines are significantly elevated as a result of having been in a stressful situation for this short time. The net fluorescence of non-stressed chicks was 50 ± 8 (s.e.) and of stressed chicks 75 ± 7 (s.e.). The difference is significant at $P < 0{\cdot}05$. Since we did not measure catecholamines in peripheral blood, the source of their increase in brain is uncertain. No doubt, sympathetic activation with concomitant adrenal medullary release could account for at least some of the amines. It is an intriguing notion that the lack of a blood-barrier to these naturally occurring depressant compounds in immature organisms might have survival benefit in species which exhibit "freezing" under stressful situations or where sedation would allow greater resistance to environmental changes.

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